

THE INDUCTION OF ENLARGEMENT OF RAT
HEPATOCYTE NUCLEI BY CERTAIN TOXIC CHEMICALS

M.R. JACKSON B.V.M.S. M.R.C.V.S.



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A B S T R A C T

This thesis is concerned with the pathological enlargement of hepatocyte nuclei by increases in DNA content.

The experimental work confirms the mechanism by which carbon tetrachloride brings about this effect and provides novel and detailed information on nuclear enlargement induced by dimethylnitrosamine and thiocetamide. The significance of this effect, which has been shown to be induced by many hepatotoxic procedures, is given a broad appraisal.

Variation in nuclear ploidy from birth onwards is reviewed. It becomes apparent that the first three weeks of life is an ideal period for studying the effects of toxins on nuclear enlargement. Attention is then given to toxic substances which induce nuclear enlargement in the liver and the mechanisms which underly this phenomenon. From this appreciation there emerges three groups of hepatotoxic chemicals: i) those inducing increased nuclear ploidy during an elevation of mitosis ii) those inducing increased nuclear ploidy during a depression of mitosis and iii) those whose action on the cell cycle is, as yet, obscure so that no firm conclusions about them can be drawn. The hepatotoxic substances which bring about nuclear enlargement during a depression of mitosis are also capable of inducing neoplasia in the liver, and of interaction with DNA. The possible association of these three effects is briefly alluded to at this stage.

Dimethylnitrosamine, carbon tetrachloride and thiocetamide all produce enlargement of hepatocyte nuclei and the mechanism by which

they do so was investigated by microspectrophotometry, mitotic counts, binucleate counts and autoradiography.

A strain susceptibility of the rat to dimethylnitrosamine was discovered as a result of which definitive experiments were restricted to Ash/Wistar stock. A single injection of 10 mg DMN/kg body weight into 2-day old rats evoked an irregular patchy necrosis associated with central veins. This effect was found to be most pronounced towards the hilus of the organ at this dosage. At 20 mg/kg a diffuse necrosis throughout the organ was observed. The dimethylnitrosamine treatment at both levels of administration also markedly depleted hepatic haemopoietic tissue over the first 48 hours but did not effect other haemopoietic centres. It was concluded from this that necrosis of haemopoietic cells was caused by a toxic metabolite of DMN formed in the liver.

The administration of 10 mg DMN/kg to 2-day old rats produced an immediate rise in nuclear ploidy so that hyperdiploid nuclei increased from 5 percent to 35 percent by day four following injection. Over the next 17 days the proportion of hyperdiploid cells decreased to approximately 25 percent. Mitotic activity of the hepatocytes was considerably depressed in DMN treated animals over the first four days of the experiment although an increase in DNA synthesis in hepatocytes occurred over the first two days following injection. The binucleate hepatocytes were not involved in this ploidy increase and it was concluded that dimethylnitrosamine brought about a blockage in the G_2 stage of the cell cycle. Moreover, a single injection of dimethylnitro-

samine induced blockage in one cell cycle only; subsequently, normal mitoses were resumed. Further work showed that the increased nuclear ploidy induced by dimethylnitrosamine is proportional to the number of hepatocytes stimulated to enter DNA synthesis and that the antimitotic effect persists for at least four days following a single administration. Repeated injections can lead to 'megacytosis' of hepatocytes due to continued blockage of the cell cycle in G_2 during successive attempts at division.

Additional autoradiographic experiments afforded evidence that there are two populations of hepatocytes in the liver having a short and long generation time respectively. Once an hepatocyte enters the 'proliferative pool' it undergoes a number of divisions in a relatively short space of time. Although hepatocytes with generation times of at least six months were seen in both dimethylnitrosamine treated and control rats dosed at birth, they were, nevertheless, present in significantly greater numbers in the dimethylnitrosamine group at the end of this period. It was concluded however, that no particular significance could be attached to this finding with regard to the carcinogenic effects induced by the compound.

With carbon tetrachloride, there is a gradual development of susceptibility of the liver to necrosis from birth onwards. This is related to the development of processing enzymes so that by four days of age a maximum necrotoxic response is seen. Injection of carbon tetrachloride to the 4-day old rat induced a reparative wave of mitosis but no increase in ploidy. At this age there are negligible numbers of

binucleate hepatocytes. At 28 days of age, however, binucleate hepatocytes account for 30 percent of the parenchymal cell population and a reparative wave of mitosis induced by carbon tetrachloride leads to a sharp drop in the numbers of binucleate cells and a marked increase in nuclear ploidy. From this and other histological evidence it was concluded that carbon tetrachloride brings about an increase in nuclear ploidy via an intermediate binucleate system. That is, the two nuclei of a binucleate cell enter mitosis at the same time but form a common metaphase plate. Two mononucleate cells or one binucleate cell with nuclei of the next ploidy level are then formed.

The same mechanism was found in the case of thiocetamide but the wave of mitosis induced was very marked in relation to the relatively small amount of necrosis induced and may not therefore be entirely related to repair.

On this evidence it was concluded that there were two mechanisms of pathological nuclear ploidy increase in the liver. No histological evidence was found for processes other than the two outlined above viz: blockage in G_2 of the cell cycle or an intermediate binucleate cell.

A final carcinogenic experiment showed that carbon tetrachloride injected into the 4-day old rat failed to induce neoplasia after 17 months. Injection of dimethylnitrosamine into the 2-day old rat however, resulted in tumours of the kidney, liver and lung over the same period.

The above results were taken to be in agreement with the initial postulate that substances with an antimitotic activity on liver parenchymal cells were also likely to induce neoplasia in the liver. Circumstantial evidence indicating that damage to DNA might explain the antimitotic action of these hepatotoxic substances is discussed. Other mechanisms such as inhibition of protein synthesis are also mentioned. Finally, the ability of mammalian cells to repair damage to DNA is also discussed as a possible factor in the varying persistence of the antimitotic action and of other cell cycle effects of some hepatotoxic procedures.

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CHAPTER I
THE IMPORTANCE OF THE LIVER IN
TOXICOLOGICAL STUDIES

From numerous studies carried out on the physiological processes and pathological responses of the liver, it has emerged that this organ has many diverse and specialised functions to perform. These include bile secretion, fat, protein, carbohydrate and iron metabolism, vitamin metabolism and storage, and both endogenous and exogenous detoxification.

It is not surprising that the main metabolic pathways concerned with foreign compound metabolism are sited in the liver since, in the normal course of events, most foreign agents gain access to the body via the alimentary tract. The liver is the first organ through which blood from the intestinal venous system must pass, and therefore, in this position it acts as a major filter to substances absorbed from the gut. The biochemical pathways in the liver for the transformation and conjugation of foreign substances ensure that many potentially harmful agents are speedily eliminated from the body. In performing this function however, the liver is especially vulnerable to the injurious effects of many compounds. It is perhaps ironical that in the process of metabolising some substances, the liver converts what was a relatively harmless compound into a potent hepatotoxin capable of bringing about cell degeneration or neoplasia.

Toxicologists have come to view the liver as an organ which can yield very valuable information on cellular responses to injurious agents. From such studies it has been found that many chemical and physical treatments can result in profound biochemical and morphological changes in the liver.

One such morphological change which is seen following various hepatotoxic procedures has been an increase in parenchymal cell size coupled with an increase in nuclear size. A broad review and investigation of the possible mechanisms by which hepatocytes increase in size following hepatotoxin administration is an aspect of liver morphology which has not, as yet, been undertaken. It was felt that such a study would be valuable in gaining a more comprehensive appreciation of pathological hepatocyte enlargement when viewed in the light of the physiological variation in cell size seen normally in the liver.

As has been illustrated by biological science many times in the past, manipulation of cellular processes by administration of toxic agents often provides valuable information of both the normal and pathological processes in cells and tissues. This thesis deals very much with the immediate effect of carcinogenic and/or necrotoxic chemicals on the cell cycle and in so doing attempts to give a more comprehensive explanation of the action of these compounds on cellular proliferation in the liver.

Specifically, this thesis is concerned with the nature and possible significance of nuclear enlargement and concomitant increase

in DNA content of liver parenchymal cell nuclei following administration of hepatotoxins. Nuclear size increase not accompanied by increase in DNA content is not considered in any detail. Extreme variation exists normally in adult mammalian liver with regard to cell and nuclear size and this is related largely to the ploidy of the cell. Ploidy may be defined as the number of multiples of the haploid number of chromosomes which any cell contains in either a mono- or a multinucleate state. Polyploidy of a cell represented by more than one nucleus, each of which contains the diploid or above chromosome number, will, hereafter, be referred to as cellular ploidy. On the other hand, variation of chromosomal content within individual nuclei whether or not more than one nucleus is present in the cell will be termed nuclear ploidy.

The next chapter of the introduction deals with the changes in the pattern of hepatic cell ploidy from birth to maturity. Most work on ploidy development has been performed with rats and the literature reviewed below thus deals with this species unless otherwise specified. From this survey are indicated the factors which led to the choice of the neonate rat as an experimental animal throughout much of this work.

The third chapter reviews the broad spectrum of hepatotoxic factors which have been observed to bring about nuclear enlargement in liver parenchymal cells with emphasis being placed on the possible mechanisms by which they would appear to bring about such increases in size.

For most of the experimental work, two compounds with essentially differing actions on the liver, were selected from these hepatotoxic factors and the mechanism by which they bring about nuclear enlargement investigated. In the fourth chapter these two compounds are comprehensively reviewed and the classification of dimethylnitrosamine as a necrotoxic and carcinogenic agent justified, in contrast to carbon tetrachloride which has a predominantly necrotoxic action.

CHAPTER 2
THE DEVELOPMENT OF POLYPLOIDY
IN NORMAL LIVER

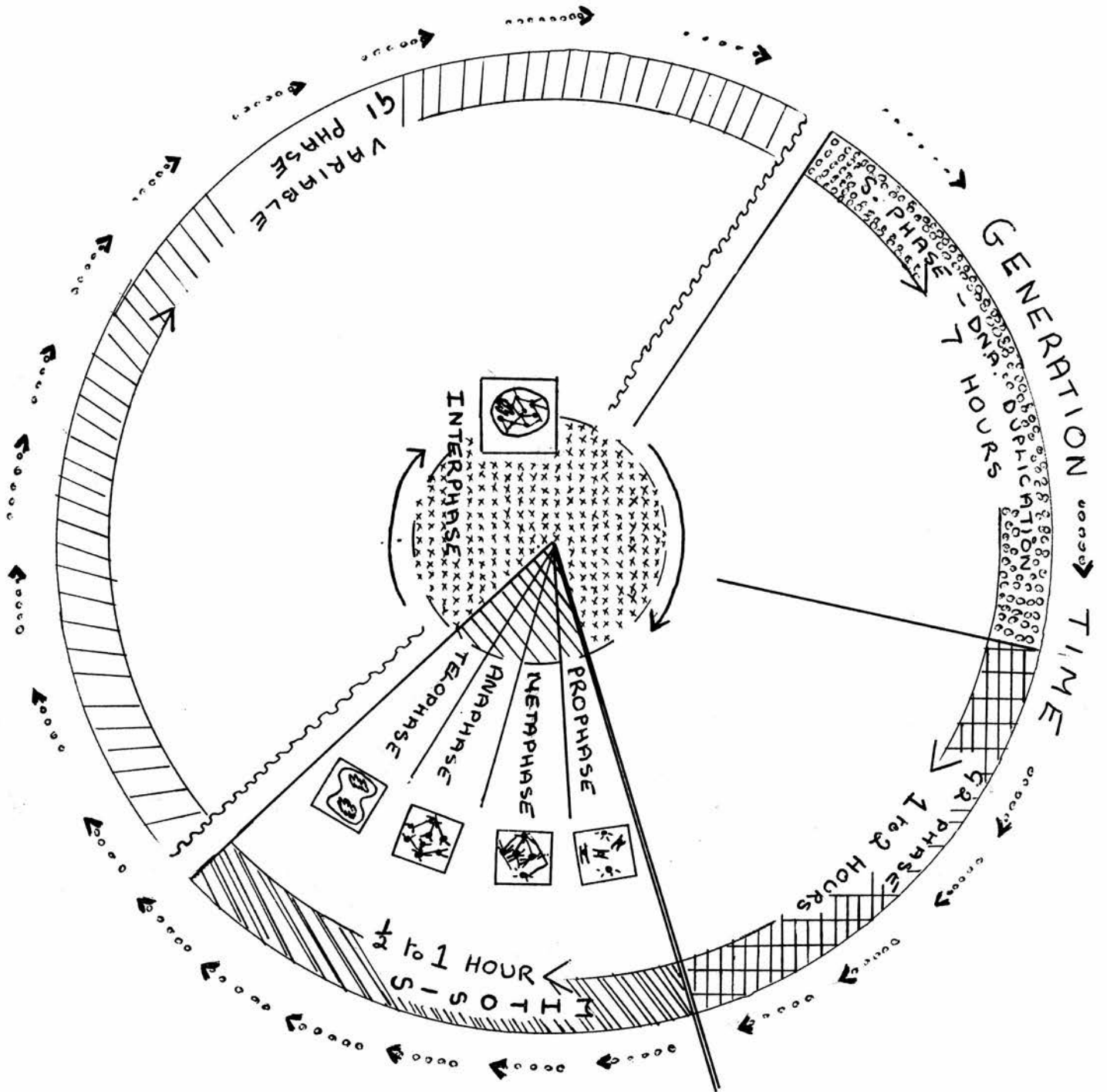
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The adult mammalian liver consists, to a large extent, of a mixed population of mono- and binucleate cells, the nuclei of which can be further divided into varying ploidy classes. At birth this population is almost exclusively mononucleate and diploid. The adult state is reached by approximately the eighth week of life in the rat. The literature concerning the mitotic activity, binucleate cell formation and nuclear polyploid development which lead to the adult hepatocyte population is reviewed below.

Much of this work deals with the passage of the cell through the cell cycle which has been divided into four major stages (Fig. 2.1) by recognition of the two major events of DNA synthesis and mitosis (Ham and Leeson 1950a).

The time between successive mitoses is termed interphase. In most cell populations the duration of DNA duplication, G_2 and mitosis have been found to be approximately 7 hours, 1-2 hours and $\frac{1}{2}$ -1 hour respectively (Cameron 1971). The duration of G_1 varies considerably from one cell type to another and is thus responsible for varying generation times between different cell populations.

FIG. 2.1



1) Mitotic activity in normal liver growth

The growth of the liver from birth to weaning is brought about to a great extent by the mitotic activity of the parenchymal cells with a consequent increase in the total number of hepatocytes. Later in life, as will be described, the formation of binucleate and mononucleate polyploid cells plays a major part in increases in liver size without concomitant increase in cell number.

The liver develops in the embryonic stage from an outpouch of epithelial endoderm from the intestine (Ham and Leeson 1950b). The cells which are closest to the site of origin of this diverticulum then develop into tubules, the early bile ducts, while those cells most remote from the original outgrowth form clumps and plates of hepatocytes. Sinusoidal development takes place between these plates of cells.

At birth (McKellar, 1949) the liver is seen histologically as a more or less flat sheet of hepatocytes; scattered throughout there are large islets of haemopoietic tissue which become progressively smaller until their eventual disappearance in approximately the third week of life.

McKellar (1949) described the further development of the liver as a branching of the central veins with mitotic activity of hepatocytes between these branches so that, by the end of the first week of life, a lobular pattern had been established. In terms of liver weight, the growth rate is rapid over the first eight weeks of life, after which it slows considerably showing an exponential decline with

body weight (McKellar, 1949; Post and Hoffman, 1964; Doljanski, 1960).

The mitotic activity of the parenchymal cells in the liver presents a parallel picture to that of weight increases of the liver, the former being greatest in the first eight weeks of life and showing an exponential decline when plotted against body weight (McKellar, 1949; Tier and Ravanti, 1953; Givol, 1957; Doljanski, 1960; Nadal and Zajdela, 1966). After the eighth week of life, mitoses are seldom encountered and their occurrence has been put as low as 1 per 10,000 - 20,000 cells in adult rat liver (Brues and Marble, 1937).

The distribution of mitoses throughout the liver has been reported to vary with age. McKellar (1949) found them randomly distributed during the first week of life when mitotic activity was relatively low; they then had an intermediate zonal distribution in the lobule until the seventh to eighth week of life over which period mitotic indices were observed to be high. The low mitotic activity in the liver of the adult makes a study of their spatial distribution very difficult. Although Milne in 1909 had formed an opinion that mitoses in rat liver tended to be concentrated in the outer third of the lobule, Brues and Marble (1937) found a random distribution both in lobar and lobular sites. Their work was supported by McKellar (1949).

At first sight more recent literature concerned with the diffuse liver regeneration following partial hepatectomy (Grisham, 1962; Fabrikant, 1967) and acute carbon tetrachloride damage (Melvin, 1968) tends to confirm the early work of Milne on the spatial distribution of mitotic activity. However, extrapolation from the regenerating to

the normal liver does not appear to be valid. Autoradiographic studies of hepatocyte nuclei which have incorporated tritiated thymidine during DNA synthesis, produce a higher index of potential cell division than do observations of actual mitoses in the liver (MacDonald, 1961). The spatial distribution of labelled hepatocyte nuclei within the lobule is thus more easily evaluated, and Schultze and Oehlert (1960), MacDonald (1961), Grisham (1962), and Fabrikant (1967a), have all reported that when this method is utilized the nuclei which take up the radioactive thymidine appear to be randomly distributed. In contrast, the same labelling system in reparative growth following partial hepatectomy has shown a periportal distribution of cells in DNA synthesis, (Fabrikant, 1967a; Butcher and Swaffield, 1964; Grisham, 1962). The last of these authors suggested that the peripheral portions of the lobule represent a reserve zone of growth but that under normal conditions the hepatocyte mitoses are randomly distributed.

The above studies involved experimental systems which, as it were, 'photographed' mitotic activity over a very short period of time and no conclusions could be drawn with regard to the replicating pattern of the cells.

Utilising the autoradiographic technique of Quastler and Sherman (1959), and counting labelled mitoses and grain counts on interphase nuclei at several time intervals after injection of 1 $\mu\text{c/gm}$ body weight of tritiated thymidine, Post et al. (1963) were able to estimate the average replicating time of hepatocytes in the three week old rat.

Very soon after this, Post and Hoffman (1964) investigated the nature of this replicating pattern at various ages and showed that the total number of cells engaged in division (proliferating pool) decreased very considerably with age. From three weeks of age onwards the replication pattern of the hepatocytes was clearly separable into distinct cell cycles. In the one day old rat, however, such separation of successive cycles was not easily discerned. Later work (Post and Hoffman, 1965a) confirmed this finding in the one day old rat liver and also demonstrated that the replication time was considerably shorter at this age, than in the three and eight week and also the six month old animals. This indicated that it was the extreme rapidity of the cell cycle of hepatocytes shortly after birth which accounted for the somewhat confused picture of replication which was obtained at this age. In this connection it is of interest that the replicating time of hepatocytes has also been observed to be relatively shorter in regenerative growth of the liver (Fabrikant, 1968). Post and Hoffman (1965a) were also able to show that the replicating cells are almost exclusively formed from the diploid cell population throughout life although this work gave no indication of any differentiation between mono- or binucleate cells of this ploidy class. It was suggested, moreover, that the decreasing numerical size of this cohort of replicating cells with age was through serial activation of progressively smaller groups of cells in the proliferating pool. In this way, random aggregates of cells would be involved so that when one group had completed its prescribed number of cycles, a new and smaller group would be activated.

With the above kinds of variations in the liver, values from studies indicating the life span of liver cells should, it is felt, be regarded with caution. MacDonald (1961) estimated the average life span of parenchymal cells in normal liver to be in the order of 191 to 453 days. On the basis of dilution of nuclear label, however, he could, from his autoradiographic observations, divide the hepatic cells into two populations, one of which divided or died within sixty days and the other which survived for at least six months. Generation times and duration of the various stages of the cell cycle have, moreover, been shown to increase with age (Post et al., 1963; Post and Hoffman, 1964; Post and Hoffman, 1965a) and to decrease in restorative growth in the liver (Fabrikant 1968).

Two other major factors influence cell proliferation in the liver, the first being intrinsically hormonal and the second, extrinsic, being concerned with circadian rhythms. There is much evidence, however, that the latter variations are in themselves mediated through a humoral mechanism.

Anterior pituitary growth hormone is necessary for normal growth rates in the liver (Simpson et al., 1949) and also for normal hepatocyte mitotic activity (Di Stephano and Diermeier, 1959). In hypophysectomised rats there is delayed onset of regeneration (Higgins and Ingle, 1939; Rabes et al., 1965; Hemmingway and Cater, 1958). Injection of growth hormone into intact mice has been shown to stimulate parenchymal cell mitosis (Litman et al., 1958).

Thyroid hormone is also associated with liver growth. Administration of thyroxin to normal rats produces an increase in mitoses while thyroidectomised animals show reduced mitoses and liver growth as compared to control groups (Carriere, 1962).

The role of the adrenal gland has also been explored and cortisone has been found to reduce mitotic activity in the liver parenchymal cells in intact rats (Horvath and Kovaks, 1956) while adrenalectomy increases mitotic activity in reparative growth. Likewise, administration of cortisone during liver restoration following partial hepatectomy diminishes the mitotic index of the hepatocytes in the first few post-operative days in rats (Horvath and Kovaks, 1956) and in mice (Roberts et al., 1952). It has been suggested that this depression of mitosis is due to cortisone suppressing the onset of DNA synthesis in the liver cells (Feigelson et al., 1962).

Finally, evidence has been published demonstrating that the cellular kinetics of the parenchymal cells of the liver are not consistent throughout the day. Two-hourly mitotic counts on three-week old rats showed mitotic activity to be at its highest level from six a.m. until twelve p.m. (Jackson, 1959), a trend also seen in weanling mice (Wilson, 1948; Litman et al., 1958; Barnum et al., 1958) and in rats following partial hepatectomy (Jaffe, 1954). Investigation of DNA synthesis in hepatocytes has also shown variation throughout the day with relatively high activity from four until seven a.m. (Messier and Leblond, 1960; Fabrikant, 1967a).

From this experimental evidence, it is clear that the growth rate and mitotic activity are highest in the rat liver during the first eight weeks of life. In this period and when the liver is undergoing reparative growth and mitotic activity is also high, hepatocyte proliferation tends to be concentrated in the outer third of the lobule. In the adult rat, when the mitotic index is relatively low, mitoses are randomly distributed. Replicating pools of parenchymal cells exist in the liver, the cells of which can divide repeatedly in a short space of time, a facet which makes the life-span studies of the hepatocytes difficult to evaluate. This overall basic pattern of cell division is modified considerably by hormonal factors and a diurnal rhythm.

2) Binucleate cells in the liver

The ploidy of a cell is the number of multiples of the haploid number of chromosomes which that cell contains. In the mammalian liver polyploidy of a cell may be represented by a multiple number of nuclei within the cell, or by a multiple number of chromosomes within a nucleus, or by a combination of both of these possibilities. The following pages deal with the first of these examples of polyploidy, namely multinuclearity.

Light microscopy of the mammalian liver reveals that the hepatocytes are usually either mononucleate or binucleate. Parenchymal cells containing more than two nuclei have been reported in adult mice and man (Wilson and Leduc, 1948) and in the rat (Daoust, 1940) but it would appear that these are exceptional observations. The proportions

of mononucleate and binucleate cells can vary considerably from one mammalian species to another (Munzer, 1923). The rat and the mouse livers have been the most extensively studied with regard to the development of binucleate cells from birth when the parenchymal cell population is almost exclusively mononucleate, to maturation when a mixed population is found to exist. It is also mainly from this work on these two species that the theories of the mechanism by which mononucleate cells can give rise to a binucleate cell population have been published.

A number of authors have observed the development of the binucleate cell population in the rat from birth onwards (McKellar, 1949; St. Aubin and Bucher, 1952; Alfert and Geschwind, 1958; Nadal and Zajdela, 1966) and their results are in general agreement. At birth the hepatocyte population is almost exclusively mononucleate and diploid. The numbers of binucleate diploid cells increase slowly until between the second and third weeks of life when there is a sharp rise in their numbers to reach a peak between the fourth and fifth week of life. They then represent approximately thirty percent of the hepatocyte population. From this time they show a slow exponential decline until the fourth month of life when their numbers stabilise at the normal adult level of approximately fifteen to twenty percent. At the commencement of this diploid binucleate decline, tetraploid binucleate cells begin to make their appearance, so that at four months of age the total binucleate cells represent approximately twenty five to thirty percent of the hepatocyte population. These figures on the

adult binucleate cell population are further supported by Sulkin (1943), and Harrison (1953).

Descriptions of the spatial arrangement of binucleate cells within the liver lobule are conflicting. Jacoby (1925) found more binucleates adjacent to the central vein and portal tracts than in the intervening areas; Munzer (1923) placed the larger proportion of binucleate cells in the region of the central vein, as did Sulkin (1943). McKellar (1949), however, stated that he found more binucleate cells in the intermediate zone of the lobule at all stages studied from two weeks to one year. Geller (1965) made a more specific study of the problem in adult rats and observed the diploid binucleate cells to be clustered around the central vein and portal triads and along the terminal portal venules, while tetraploid binucleate cells predominated in the intervening areas. Whatever interpretation is made of the above studies it would appear that several workers consider that the distribution of binucleate cells in the liver lobule is not random. Carriere (1969) indicated that the varied results obtained by different groups of workers could be age-dependent although such a conclusion is not supported by the work of McKellar (1949).

The mechanism by which binucleate cells are formed has attracted two main hypothesis and a third which has gained less support.

Observation of constricted nuclei in hepatocytes has favoured the possibility of an amitotic method involving equal division by splitting of the nucleus, in the rat (Clara, 1931; Suppan, 1966) and in the mouse

(Wilson and Leduc, 1948). Against this hypothesis, however, a number of other experimentalists have reported their repeated failure to find sufficiently constricted nuclei which they would be prepared to accept as evidence of amitosis (Schultz et al., 1923; Brues and Marble, 1937; Beames and King, 1942; Sulkin, 1943). Furthermore, as has been pointed out by Wilson and Leduc (1948) it is impossible to decide whether constricted nuclei are in fact in the process of division or fusion. Reports on the occurrence of haploid liver cell nuclei (Marquardt and Gläss, 1957) would, however, be in keeping with the possibility of amitosis and the validity of this mechanism remains open.

A further mechanism which was proposed by Wilson and Leduc (1948) postulates the formation of binucleate cells from the fusion of two mononucleate cells. There is no direct evidence either for or against this hypothesis. These authors also proposed that polynucleate cells were formed by this process of cell fusion.

The third possibility which was put forward by Beames and King (1942) proposes the failure of cleavage of the cytoplasm at telophase as a mechanism. These workers and others, Brues and Marble (1937) in the rat, and Wilson (1942) in the mouse, observed hepatocytes in histological section which had reached late telophase with no evidence of formation of a cytoplasmic furrow. This concept of binucleate cell formation through a mitotic process is further supported by the fact that the high mitotic rate in the young rat coincides with a massive increase in the numbers of binucleate cells (McKellar, 1949), an occurrence which is difficult to reconcile with an amitotic theory.

Finally, additional evidence in favour of the mitotic process comes from experiments demonstrating that hormonal factors which influence mitotic rate in the liver of the growing rat also affect the rate of development of binucleate cells (Carriere, 1962). It should be noted that, although mitosis appears to be necessary to the formation of binucleate cells, a wave of mitosis induced in the adult liver following chemical damage (Himes et al., 1957) or partial hepatectomy (James et al., 1966) can result in a profound drop in their numbers. This finding is of importance when taking into account the mechanism of nuclear polyploid formation as will be seen in the next section.

The above work has thus shown that up to two weeks of age the liver contains negligible numbers of binucleate cells after which time there is a sharp rise to reach a maximum just after weaning at approximately four to five weeks of age. The mechanism of formation, at least in the young animal, would appear to be predominantly by failure of cytokinesis at late telophase in the mitotic cycle, although the possibility of an amitotic process cannot be completely ruled out.

3) The development of nuclear polyploidy in the liver

Nuclear polyploidy is concerned with the multiples of chromosomal content of individual nuclei. This form of polyploidy would appear to be widely spread throughout the livers of the mammalian species but once again most work has been performed with rats and mice. The main increase in nuclear size in rodent liver has been shown to be due to this multiplication of chromosomal content, but enlargement to a lesser

degree can take place through increases in other nuclear constituents. The way in which a nucleus may progress from one ploidy level to the next has been satisfactorily explained by one method at least, though the other concepts which had been proposed cannot be completely disregarded.

The pattern of nuclear ploidy development is one of increase from birth to a ploidy equilibrium which is reached by maturity. A number of factors, mainly hormonal, have been shown to influence this development from birth.

Early workers had commented on the variability of nuclear size in the adult rodent liver (Heiberg, 1907; Milne, 1909), and Jacobj (1925) measured diameters of parenchymal cell nuclei in tissue sections to find that they fell into size groups which followed an approximately geometric progression. Observations of varying numbers of chromosomes in metaphase plates indicated that the variability in nuclear size might have a positive relationship to chromosomal content (Beames and King, 1942; Bieseke, 1944) and this was finally established through measurement of nuclear DNA content by microspectrophotometry (Swift, 1950). Despite the increased accuracy of this last technique, which could place the nuclei into very distinct ploidy classes, there still remained observations of nuclei with intermediate DNA values. Swift (1950) ascribed these anomalies to nuclei which were in the process of replicating their chromosomal material preparatory to division, a suggestion initiated by the fact that most of the intermediate nuclei occurred in young rats where mitoses were frequent. In agreement with this, other workers, (Pogo et al., 1960), have demonstrated these

intermediate class nuclei in the neonate rat and in reparative growth of the liver in the adults when mitoses are also frequent (Heizer, 1955; Grundman and Bach, 1960). Looney (1960), using a combined autoradiographic-microspectrophotometric technique, was able to show that cells synthesising DNA preparatory to division showed ploidy values intermediate between any two classes.

Although there is a positive correlation between nuclear volume and DNA content (Swift, 1950; Fautrez and Laquerriere, 1957; Naora, 1957a; Epstein, 1967) the relationship is not entirely a direct one. Indeed this is not surprising since other nuclear constituents represent a greater proportion than DNA content, Amano (1967) placing the relative proportions of nuclear DNA, RNA and protein as 28.8 percent, 5.3 percent and 65.9 percent respectively. Naora (1957b) found that the DNA content of the diploid nucleus decreased slightly with age while nuclear size has been shown to increase with age (McKellar, 1949; Carriere and Patterson, 1962). This variation of nuclear size with age could be explained on the basis of an increase in the relative proportion of other constituents of the nucleus without increase in DNA content. Certainly, protein fluctuations can occur without any alteration in DNA content thus showing some degree of independence of the other two constituents (Di Stephano and Diermeier 1959). Nuclear histones, however, would not appear to be implicated in nuclear size variation related to protein increase as they maintain a constant ratio with DNA content. (Alfert and Geschwind, 1953; Meek, 1964). In this connection, recent investigations with cultured cells (mouse mastocytes and HeLa cells)

have revealed that over at least 8 generations histones are as stable as DNA and no irreversible dissociation of histone from chromatin occurs (Hancock 1969).

Protein increase during DNA synthesis has been implicated with the increase in nuclear size at this time (Carriere et al., 1961; Carriere, 1967). RNA represents a relatively small proportion of the nuclear content (Amano, 1967), much of which is concentrated in the nucleolus. Shea and Leblond (1966) have produced data indicating that the number of nucleoli in the nucleus of hepatocytes in normal rat liver is proportional to the ploidy and so it seems that under physiological conditions RNA would contribute little to increased nuclear size which is unrelated to ploidy changes. This does not necessarily apply to nucleolar involvement in pathological nuclear enlargement, as will be seen in a following section of this review. From these studies it would seem reasonable to assume that under normal conditions large increases in nuclear size are due to chromosomal replication, but that small fluctuations in size may take place due to variation in the relative proportions of extrachromosomal protein.

James et al., (1966) studied the distribution of polyploidy in normal adult rat liver and found in descending order of frequency, mononucleate tetraploid, binucleate diploid, mononucleate diploid, binucleate tetraploid and mononucleate octoploid cells. This frequency of respective ploidy classes of hepatocytes is in agreement with observations by many other workers including Alfert and Geschwind (1958) and Nadal and Zajdela (1966). These latter groups also studied

the development of ploidy from birth and reached similar general conclusions. They found that until the second to third week of life the hepatocyte population in the rat is almost exclusively mononucleate and diploid. During approximately the fourth week of life there is a sharp transition towards cells of higher ploidy with the rise in binucleate diploids, as described above. This rise is followed by a gradual accumulation of mononucleate tetraploids so that by adulthood this class is dominant. At around the third to fourth month of life binucleate tetraploid cells form a significant proportion of the population to be followed by the appearance of mononucleate octoploid cells. This pattern of development would appear to be repeated in most strains of rats though the absolute proportions and time of development of each ploidy class may differ to some degree. For example, the percentage of octoploid nuclei has been reported to vary. Thus, at one year of age Post et al., (1960) and Nadal and Zadjela (1966) found only one percent in Wistars while they constitute about 10% of the total population in both Long-Evans rats (Alfert and Geschwind, 1958) and hooded Sherman rats (Carriere, 1962) of similar age. There is evidence furthermore of the gradual accumulation of the larger ploidy classes with age, as Post et al., (1960) found that the incidence of octoploids had increased to approximately seventeen percent by two years of age in Wistar rats. Mouse hepatocyte nuclei show a similar pattern of accumulation, but the onset of polyploidy after birth is more rapid and there is a greater incidence of higher polyploids in the adult. (Epstein, 1967; Inamdar, 1958).

The mechanism of formation of polyploid nuclei which would appear to carry the majority of evidence in its favour is that put forward by Beames and King (1942). This proposes that binucleate cells precede the formation of the nuclear polyploidy at a higher level and infers that the nuclei of binucleate cells undergo simultaneous DNA synthesis and mitosis respectively. One mitotic spindle only is formed and the chromosomes come together on a common metaphase plate. Following telophase a binucleate or two mononucleate cells of the next nuclear ploidy level are formed.

In support of this proposal nuclei of binucleate cells have been observed to synthesize DNA at the same time (Messier and Leblond, 1960; Schultze and Oehlert, 1960; Edwards and Klein, 1961; Carriere, 1967). This would seem to be followed by the simultaneous appearance of both nuclei in prophase (Beames and King, 1942; Sulkin, 1943; Himes et al., 1957; Nadal and Zajdela, 1966). The condensation of the chromosomes from the two nuclei onto a common metaphase plate has been assumed as a result of counting metaphase chromosomes (Bieseke, 1944; Mortreuil-Langlois, 1958) and by subjectively evaluating the size of mitotic figures at metaphase in histological sections (Beames and King, 1942; Post and Hoffman, 1964; Nadal and Zajdela, 1966). The above observations could, however, be interpreted as evidence of mononucleate hyperdiploid cells in normal mitosis. On the other hand, there is a significant absence of reports of two mitotic figures in anaphase or telophase within the same cell. Such observations would be expected since binucleate cells form such a high proportion of the hepatocyte

population in adult rat liver. Furthermore, as has already been described, binucleate cells always precede the formation of new ploidy classes in normal liver development and factors which cause a drop in the number of binucleate cells in the liver also cause a shift to higher ploidy (Beames and King, 1942; Sulkin, 1943; Bucher and Glinos, 1950; St. Aubin and Bucher, 1952; Himes et al., 1957; Leshner et al., 1960; Nadal and Zadjela, 1966), both of which factors support the binucleate concept.

That mononucleate polyploid cells can give rise to additional cells of the same ploidy level by a normal mitotic process is evidenced by the observations of Carriere (1967) of mononucleate tetraploid cells synthesizing DNA and subsequent labelled mitotic figures. Looney (1960) also reported labelled tetraploid nuclei synthesizing DNA, increasing to octoploid size and returning to a tetraploid level with a halving of the tritiated thymidine grain count, indicating that division had taken place. Thus it seems clear that mitosis in binucleate and mononucleate cells can contribute towards the formation of further polyploid nuclei. There are, however, other theories which had been presented by other workers though they are based on less firm evidence.

Polyploid formation by fusion of interphase nuclei has been mentioned in relation to binucleate cell formation and constricted nuclei have been taken as evidence of this (Pfuhl, 1939).

Other methods which have been invoked are those which imply growth of the nucleus to the next ploidy level without dissolution of the nuclear membrane. Bieseke et al. (1942) described a condensation

of chromosomal material inside an intact nuclear membrane and described this as "endomitosis". Wilson and Leduc (1948) suggested a similar mechanism but proposed that the chromosomal material could reduplicate and divide without condensation within the nuclear membrane and called this a process of "cryptomitosis". This mechanism has been favoured by later workers (Leuchtenberger, 1954; Naora, 1957; Post and Hoffman, 1964) as an explanation of continued nuclear polyploid development in the adult where mitotic activity of the hepatocytes is low. Naora (1957a), from lengthy calculations on liver cell kinetics, suggested that the majority of nuclear polyploid formation was by this mechanism with diploid cells giving rise to further diploid cells by mitosis and ploidy levels being increased almost exclusively by cryptomitosis. Other facts stubbornly resist this conclusion however. Carriere (1957) found that between two and twenty-six hours after the injection of tritiated thymidine to three month old rats, the number of labelled cells, the majority of which were of the tetraploid class, doubled, suggesting division of the majority of cells synthesizing new DNA thus leaving few to undergo cryptomitosis as postulated by Naora. Further observations, such as those of D'Ancona (1941), of approximately octoploid numbers of chromosomes in metaphase plates may be taken as further evidence of the ability of high ploid nuclei to multiply through a normal mitotic process. In addition the idea that the low mitotic rate in adult rat liver indicates further growth and polyploid formation by cryptomitosis can be argued against in the light of the long life span of the hepatocytes (MacDonald, 1961). This suggests, that to

maintain, or to slowly add to the parenchymal cell population, few mitoses would be required.

The spatial distribution of nuclear polyploidy within the liver lobule is, like that of binucleate cells, uncertain. However, Geller (1965) in a specific study of the problem found cells with diploid nuclei to be more abundant around central veins and portal tracts while cells of higher ploidy were found most often in the intermediate zone.

Diurnal rhythm with regard to nuclear polyploidy has not been extensively studied but Jackson (1959) reported finding an increased nuclear size through the night hours in rats.

Finally, hormones have been shown to markedly effect the formation of polyploid nuclei in the rat.

Hypophysectomy in the growing rat, and consequent absence of growth hormone, significantly retards the rate of accumulation of polyploid nuclei which is normally seen at this time (Di Stephano et al., 1955). Injection of growth hormone into growing rats after hypophysectomy restored to a considerable extent the polyploid ratio (Di Stephano et al., 1955; Geschwind et al., 1960; Carriere, 1962). Growth hormone is not indispensable for nuclear polyploid formation, however, since a wave of mitosis induced by partial hepatectomy in hypophysectomised rats produced significant proportions of new polyploid nuclei (Geschwind et al., 1958). Further evidence on the effect of growth hormone has been afforded by the experiments on hereditary dwarf mice in which hepatocytic nuclei are predominantly diploid and in which the injection of growth hormone induced the formation of nuclei with higher ploidy

levels (Leuchtenberger et al., 1954). From these experiments it is clear that the anterior pituitary growth hormone influences the formation of polyploid nuclei. It is perhaps relevant to refer to the section of this review dealing with parenchymal cell mitoses and the effect of growth hormone on this parameter (p 13). A simple hypothesis would be that the growth hormone influences the mitotic process and changes in polyploidy are a result of these mitotic effects.

Thyroidectomy markedly inhibits the formation of polyploid nuclei in both young (Swartz and Ford, 1960) and adult rats (Carriere, 1955). The normal pattern of nuclear polyploid accumulation can be restored by injection of thyroxine (Carriere, 1955). It is of considerable relevance that other work has shown that the output of growth hormone is influenced by thyroid hormone (Eartly and Leblond, 1954), although experiments on hypophysectomized rats have shown that thyroid hormone can have a direct effect on nuclear polyploid formation (Geschwind et al., 1960). Once again mention must be made on the profound effect of thyroid hormone on mitotic activity which may be related to its effect on nuclear polyploidy.

The effects of the sex hormones have been investigated and Swartz et al. (1960) found that castration of rats at birth reduced the incidence of octoploid nuclei when the liver cell population was evaluated at four months of age.

Dunn et al. (1958) found a slight increase in the level of nuclear polyploidy when cortisone was administered to adult mice and Lowe and Rand (1956) reported that individual nuclei of each ploidy class showed

a decrease in their relative contents of DNA. These latter authors could not confirm the ploidy shift as seen by Dunn. Cortisone administration in the rat has been shown to inhibit the shift to higher ploidy levels seen following repair in the liver (Hoffman et al., 1955) (cf. mitotic activity).

Studies on the development of nuclear polyploid development have thus shown that hormones can have a profound effect, and the indications are that it is to some extent at least, through their influence on mitotic activity.

4) Summary

The major points on the above treatise on liver development which require to be emphasized with regard to the subsequent experimental work of this thesis, are as follows:-

For at least the first two weeks of life the rat liver has a parenchymal cell population which is almost exclusively diploid and mononucleate. The eventual development of polyploidy in normal liver would appear to be mainly through a mitotic intermediate binucleate cell system though the possibility of the occurrence of other mechanisms cannot be completely eliminated.

The opinion was formed that the neonate rat presented for the first three weeks of life a useful animal in which to investigate the effect of hepatotoxic chemicals on nuclear polyploid development, through any mechanism other than that of the binucleate cell. In a similar way, the optimum time for investigating nuclear polyploid formation through shifts in the binucleate cell population would be shortly after weaning when the proportion of binucleate diploid cells is greatest.

CHAPTER 3

HEPATOTOXIC TREATMENTS INDUCING

NUCLEAR ENLARGEMENT

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1. Introduction

Pathological nuclear enlargement with associated increase in cell size has been observed frequently in the liver following physical and chemical damage. The mechanisms by which hepatotoxic agents bring about this enlargement are reviewed below.

Very often observations on nuclear and cell enlargement following the administration of these toxic factors have been by subjective evaluation of histological sections of the liver, but in other instances a more quantitative approach has been adopted. In the previous section of this review it was seen that an understanding of normal nuclear enlargement and ploidy increase in the liver required relatively accurate estimations of the parameters of mitotic activity, DNA content, nuclearity and rate of DNA synthesis of the hepatocyte population. Pathological nuclear enlargement is examined in a similar fashion below and it will be made clear from this that the possible mechanisms by which these toxic factors bring about such a change enables one to place these agents into one of three groups on the basis of knowledge to date, viz:

- (1) Those agents that induce nuclear enlargement during an increase of mitosis.
- (2) Those agents that induce nuclear enlargement during a depression of mitosis.
- (3) Those agents with which the cellular kinetics have not been sufficiently studied for firm conclusions to be drawn.

Table 3.1 summarises the evidence to date on the effect on the cell cycle of hepatotoxic procedures which bring about nuclear enlargement in the liver.











Although this classification partially clarifies the mechanism by which nuclear enlargement is brought about in the liver, it does not detail the initial underlying sub-cellular damage initiating changes by some of the toxic agents. In the instance of mitotic inhibition recent work indicates that this could be caused by specific damage to the nucleus, so further comparisons are made with these aspects in mind.

Finally, the necrotoxic and carcinogenic potential of agents from each of the above groups are reviewed and it will be seen that, although necrotoxicity is a property of chemicals from all three groups, the tumourigenic property is confined mainly to the group of mitotic inhibitors. On this basis conclusions are drawn with regard to the possible association of the mechanism of nuclear enlargement with nuclear damage and carcinogenicity.

The two compounds, dimethylnitrosamine and carbon tetrachloride, which were chosen for use in much of the experimental work of this thesis as representing essentially differing types of action in the liver, are dealt with only briefly in this section since a more comprehensive survey of these two compounds concludes the literature review. (p57).

Fig. 3.1

Action on the liver of various treatments which induce enlarged hepatocyte nuclei

Treatment	Rate of DNA synthesis		Mitotic activity		Binucleate population	Ploidy	Nuclear damage			Neurosis	Carcinogenic potential
	Agent alone	Agent + part Hep.	Agent alone	Agent + part Hep.			Chromosome defects	Mutation	Biochem (interaction with nucleic acids)		
Partial hepatectomy	+		+		-	+					
Carbon tetrachloride	+										very weak
Radiation damage	-		-			+	+	+			very weak
Aflatoxin	-		-			+	+	+	+	periport	very potent
Dimethylaminoazobenzene	-					+	+	+	+	centrilob	potent
Pyrrolizidine alkaloids	-	+	-			+	+	+	+	centrilob	weak
Dimethyl-nitrosamine							+	+	+		very potent
Thiocetamide	+								+		weak
Ethionine									+	Not neurotoxic	weak
N-2-aceto-aminoflourene									+	Not neurotoxic	potent
Cyclohexamide	-		-								

Key

+ increase

Not known

- decrease

- decrease

results established or confirmed by this thesis

Polyploidy inferred from histological observation of enlarged hyperchromatic nuclei.

+?

2. Mechanisms of pathological nuclear enlargement

i) Nuclear enlargement induced during a wave of mitosis

There are two deleterious factors which have been shown to induce nuclear enlargement in the liver following a wave of mitosis, the first being purely physical damage and the second chemical.

The role of partial hepatectomy in the induction of increased nuclear ploidy has already been mentioned in Section I of this review. To reiterate briefly, it has been demonstrated that partial hepatectomy in the adult rat increases nuclear polyploidy with an associated fall in the level of binucleate cells (James et al., 1966). On the other hand, there was no rise in nuclear polyploidy of seven-day old rats following partial hepatectomy. Binucleate cells are few in animals of this age (Nadal and Zadjela, 1966).

Chemical damage, as induced by prolonged administration of carbon tetrachloride at necrotoxic levels, has been reported to increase the size of parenchymal cells in the liver of the mouse (Eschenbrenner and Miller, 1946). The increase in nuclear polyploidy in the rat liver following a single necrotoxic dose was quantitatively demonstrated (Hoffman et al., 1955; Hoffman et al., 1956) and shown to persist for some weeks. A later study (Himes et al., 1957) demonstrated that the increase in nuclear polyploidy following multiple necrotoxic doses of carbon tetrachloride in the adult rat was accompanied by a fall in the number of binucleate cells. These workers postulated that the mechanism by which carbon tetrachloride increased nuclear ploidy was through an intermediate binucleate cell system, though other possibilities such

as nuclear fusion could not be excluded. The questionable carcinogenic effects of long term carbon tetrachloride administration are fully dealt with in Chapter 3.

ii) Nuclear enlargement induced during a depression of mitosis

Hepatotoxic factors which have been shown to depress mitosis and increase nuclear and cellular size in the liver can again be divided into physical and chemical categories.

The only physical factor involved is ionizing radiation and there are two experimental systems in which it has been observed to affect liver cell nuclei.

The first of these systems involves external x-irradiation of the whole animal or organ. There is considerable evidence that radiation damage to the liver following partial hepatectomy results in a depression of DNA synthesis, delay in the onset of the reparative waves of both DNA synthesis and mitosis, and aberrant mitoses (Fabrikant, 1967b; Fabrikant, 1969). The latter of these studies showed that the quantitative nature of each of these effects depends to a large extent on which stage of the cell cycle the cell has reached when exposed to radiation. However, there are also reports that radiation damage can induce enlargement of hepatocytes and their nuclei through inhibition of mitosis. Thus, when this treatment is given either before or after partial hepatectomy, the restorative increase of mitosis is both delayed and depressed. At the same time there is a concurrent increase of nuclear size which is greater than that of the partially hepatectomised control rats (Williams, et al., 1952; Cater et al., 1956; Leong et al.,

1961; Webber and Stitch, 1965; Fabrikant, 1969) and mice (Fabrikant, 1967b). Liver weights do not differ significantly between the two groups however (Gershbein, 1956), thus providing an indication that increased ploidy and cell size in the irradiated group is equivalent to increased cell number in the control rats. Outwith the liver, it is interesting that x-irradiation of HeLa cells in culture has been shown, at certain doses, to induce a profound inhibition of mitosis with the formation of giant cells containing grossly enlarged nuclei (Puck and Marcus, 1956). Nor is the in vivo effect of x-irradiation limited to the liver, since intestinal epithelial cells of mice have shown a lengthening of the G₂ period of the mitotic cycle, a further indication of at least the delayed onset of mitosis (Lamerton, 1966).

The second system in which radiation damage, in this case β radiation, has been shown to induce nuclear enlargement in the liver, involves incorporation of tritiated thymidine into the nucleus. Below a certain level, this radioactive compound would seem to have no significant effect on cell kinetics and has been used extensively to study cell proliferation e.g. Quastler and Sherman (1959). Post and Hoffman (1961), however, showed that doses at and above 2 $\mu\text{C/gm}$ body weight administered to three week old rats resulted in an increase in nuclear ploidy when compared with appropriate controls. A significant increase in ploidy was seen within twenty-four hours and, moreover, this increase continued in extent for at least a further two weeks. Later work by these authors (Post and Hoffman, 1965b; Post and Hoffman, 1967) has confirmed that the cell cycle is considerably disrupted when H³

thymidine is administered at relatively high levels of (2-10 $\mu\text{c/gm}$ body weight), with delay in passage through the G_2 phase, inhibition of mitosis and a doubling of the generation time due to a lengthening of the G_1 interval. It was concluded (Post and Hoffman, 1967) that the nuclear ploidy increase suggested an arrest of the cell cycle after DNA synthesis with failure to enter mitosis.

Besides the physical agents mentioned above there are a number of chemicals which give rise to enlarged parenchymal cells and nuclei and which also appear to act through a depressive activity on mitosis.

a) Aflatoxin

The isolation of a toxic factor from peanut meal (Schoental, 1961) and outbreaks of liver disease in domestic animals (Blount, 1961; Loosemore and Harding, 1961; Asplin and Carnaghan, 1961) later led to the incrimination and identification of a metabolite of the mould Aspergillus flavus as the causative agent. The metabolite aflatoxin has since been separated into distinct components which are known as B_1 , B_2 , G_1 and G_2 (Nesbitt et al., 1963). Butler (1964) observed grossly enlarged parenchymal cells with hyperchromatic nuclei following a single necrotoxic dose of Aflatoxin B_1 , to the adult rat. Although the cellular kinetics following a single dose of any of the Aflatoxins have not been extensively studied as yet, De Recondo, et al., (1965) have shown an inhibition of DNA synthesis by Aflatoxin B_1 following partial hepatectomy. In addition, Rogers and Newberne (1967) have demonstrated a depression of mitotic activity and thymidine uptake following a single dose of Aflatoxin B_1 . In vitro studies of the

effects of Aflatoxin B₁ on HeLa cells (Harley et al., 1969) and human embryonic liver cells (Zuckerman, et al., 1968) have also demonstrated a profound inhibitive action of the toxin on DNA synthesis. In these studies the former authors postulated that, since Aflatoxin interacts with DNA, it is possible that the toxin inhibits DNA synthesis by combining with DNA and thereby preventing transcription by DNA polymerase. Direct damage to polyribosomes by the toxin was also indicated in this work.

a) Amino-azo compounds

4-dimethylaminoazobenzene (DAB) first attracted the attention of toxicologists by its carcinogenic properties (Kinosita, 1936) but Orr (1948) was the first to report the enlarged hepatocytes with hyperchromatic nuclei seen after administration of the compound.

The mechanism by which this cytoplasmic and nuclear enlargement is brought about has been indicated by the results of observations of the action of DAB on the cell cycle. Partial hepatectomy and concurrent administration of DAB to the rat has revealed an inhibition of mitosis during the restorative phase when compared with partially hepatectomised control rats (Brody, 1960; Banerjee, 1965). Similar findings have been reported in rats fed the closely related compound 3'-methyl-4-dimethylaminoazobenzene and subjected to partial hepatectomy (Stitch and Maini, 1962). On the other hand, if intact rats are fed DAB over a long period then there is an increase in hepatocyte mitosis (Daoust, 1962). This observation of Daoust is difficult to reconcile with the results obtained following partial hepatectomy. It would seem that in long-term

administration the antimitotic effect of the compound is lost. In this connection, a more recent study by Simard et al. (1968) found that the effect of DAB on the cell cycle varies with the time over which the carcinogen is fed. In the early stages of DAB feeding, termed preneoplastic by these authors, there was an arrest or delay of the parenchymal cells through the G_2 phase of the cell cycle and at the same time an increase in nuclear ploidy levels. In the neoplastic tissue which developed later, however, the mitotic activity increased while ploidy values of the hepatocytes decreased. It was postulated from these studies that in normal liver tissue a strong block exists in both the G_1 and the G_2 stages of the cell cycle thus giving rise to the low mitotic activity normally seen in adult liver. Furthermore, it was suggested that in preneoplastic tissue, the block at G_2 increases in degree, giving rise to higher ploid nuclei and mitotic inhibition (as was also observed by the work mentioned above involving simultaneous DAB administration and partial hepatectomy). In neoplastic tissue however both the G_1 and the G_2 blocks were decreased and this accounted for the increased mitotic activity and fall in nuclear ploidy observed by Simard and co-authors, in hepatomas induced by DAB.

c) Pyrrolizidine alkaloids

Without doubt the most striking enlargement of liver parenchymal cells and their nuclei occurs following the administration of many of the pyrrolizidine alkaloids. The development of these enlarged cells is accompanied by gross distortion of the liver architecture (Selzer et al., 1951; Bull, 1955; Schoental and Magee, 1957; Bull and Dick,

1959). It was Bull in 1955 who first coined the term 'megaloocyte' to describe the grossly enlarged hepatic cells. Moreover, it has been established that a single dose of the alkaloid lasiocarpine administered to the adult rat is sufficient to give rise to the progressive accumulation of megaloocytes in the liver (Schoental and Magee, 1957; 1959; Nolan, et al., 1966). The nature of the development of enlargement was suggested by Schoental and Magee (1959) to be through an inhibition of mitosis. This inhibition is not absolute, however, as these authors and others (Peterson, 1965; Jago, 1969) observed megaloeytic mitotic figures, although many were abnormal in configuration.

The involvement of mitotic inhibition in cell enlargement is further indicated by the dose-and-age response relationship to the pyrrolizidine alkaloids. In circumstances where division of hepatocytes is normally frequent, such as in the young rat, (Schoental and Magee, 1959; Schoental, 1959; Nolan et al., 1966) or following partial hepatectomy in the adult rat (Peterson, 1965), administration of pyrrolizidine alkaloids produced almost immediate gross megaloeytosis. On the other hand, when administered to the adult rat, where mitotic activity is low, the development of the lesion is considerably delayed unless the compound is given at a necrotoxic level (Schoental and Magee, 1957; Bull and Dick, 1959; Schoental and Magee, 1959). In a more recent study, Jago (1969) confirmed the quantitative aspect of this mitotic inhibition and found, in agreement with the work of Peterson (1965), that the stimulus for regeneration and DNA synthesis in the parenchymal cells was not significantly altered, the increase in cell size being induced by failure

of the cell to enter mitosis. It therefore seems apparent that the rate and degree of the megalocytic lesion depends on two factors: firstly the number of cells in which mitotic inhibition is induced and, secondly, the extent of stimulation of cells to enter division through either normal growth or regeneration following damage to the organ.

Theoretically there are several possible fates open to megalocytes, none of which have been established. Observation of degenerative forms of megalocytes has prompted workers to suggest that it is primarily a non-viable form of cell (Schoental and Magee, 1957; 1959; Bull and Dick, 1959; Nolan et al., 1966). In the later publication Schoental and Magee (1959) also commented on the abnormal mitoses observed in their preparations, suggesting that some of these cells might be viable and possibly connected with the carcinogenic effect which was seen when pyrrolizidine alkaloids were fed to rats (Cook et al., 1950). Recent observations with the electron microscope have supported the view that this type of cell is capable of survival (Schoental, 1968) and other authors have also postulated a connection between this cell type and eventual tumour formation (Dybing and Erichsen, 1959; Scheuer, 1963). The fact that mitotic figures are observed in these megalocytic cells has been taken as an indication of at least the partial reversal of the mitotic inhibition (Jago, 1969). Moreover, the finding of nodular parenchymal regeneration in the absence of continued administration of alkaloid (Bull and Dick, 1959) indicates that some cells completely escape or recover from the mitotic inhibitive effect and cell clones, capable of normal and even compensatory growth, are able to develop.

d) Cyclohexamide

There is one further compound which requires to be mentioned in relation to mitotic inhibition in the liver. Verbin et al., (1969) found that cyclohexamide, an antibiotic, could interfere with the passage of hepatocytes through both DNA synthesis and mitosis. Specifically, if injected 12, 22 or 24 hours after partial hepatectomy (corresponding to G_1 , S and G_2 respectively in the partially synthronised cell population) mitosis was grossly depressed 26 hours post-operatively when compared to appropriate controls. Furthermore, analysis of DNA synthesis 24 hours after partial hepatectomy and two hours following the injection of cyclohexamide showed an inhibition of uptake of tritiated thymidine of approximately 98 per cent. Incorporation of Leucine- C^{14} was also evaluated two hours following the injection of cyclohexamide which in turn was injected at a time when the majority of the cells were expected to be in the G_2 stage of the cell cycle. The uptake of this labelled amino acid was also inhibited by some 95% and on this basis Verbin et al., postulated that it was the inhibition of protein synthesis which brought about the observed block in G_2 of the cell cycle. Nuclear ploidy was not measured and only one mitotic count was recorded 26 hours post-operatively. Thus, the inhibition might well have been merely a delay in the onset of mitosis and DNA synthesis rather than an absolute depression resulting in a shift in ploidy. Such delay in the onset of both DNA synthesis and mitosis is a well known phenomenon following radiation damage (Fabrikant, 1969). Certainly the effect of cyclohexamide on the kinetics of intestinal

epithelial cells is overwhelmingly one of delay in the passage of cells through G_2 rather than an absolute block followed by increased ploidy (Verbin and Farber, 1967). However, the possibility that cyclohexamide might induce increased nuclear ploidy via mitotic inhibition cannot be discounted entirely and this concept is dealt with further in the concluding discussion.

iii) Unconfirmed mechanisms

The last group of compounds to be discussed are those hepatotoxins which bring about enlargement of parenchymal cells and their nuclei but with which cellular kinetics following dosage have not as yet been studied in detail. As a consequence, no real conclusions have yet been reached concerning the manner in which they bring about such an effect.

a) Dimethylnitrosamine (DMN)

Early studies on the acute effect of DMN on the rat liver (Barnes and Magee, 1954) revealed a significant increase in nuclear size of the parenchymal cells of the liver within seventy-two hours of a necrotoxic dose. A more quantitative approach has since indicated (Christie and LePage, 1961) that the increase in size is related to increased ploidy as judged by measurement of nuclear size and photometric estimation of DNA content during prolonged administration of DMN. These workers also commented that mitotic activity was more frequently observed in the liver sections of DMN treated animals than in those of controls. Grundman (1967), in a study on nuclear ploidy following administration of diethylnitrosamine after partial hepatectomy, found a decrease in DNA synthesis and nuclear polyploidy as compared with operated controls.

b) Thiocetamide

It has been observed that marked nuclear enlargement, as evaluated by diameter measurement, took place within two days of the commencement of administration of this compound (Rather, 1951). Biochemical estimation of the DNA content of the liver (Thomson et al., 1953) failed to reveal that this nuclear enlargement resulted in an absolute increase in total DNA of the liver. Carnes (1953) and Heizer (1955), however, both confirmed the karyometric findings of Rather (1951) and showed that a positive relationship existed between increased ploidy and nuclear volume. Ploidy was measured by a microspectrophotometric technique in these later studies. The relationship would not appear to be exact since Bernstein et al. (1953) observed that nuclear size determinations did not conform closely to a geometric series (as occurs in normal liver) indicating perhaps that another nuclear constituent, in addition to DNA, was involved in the nuclear enlargement. This view was further strengthened by the photometric volume measurements of Kleinfeld (1957) who found that withdrawal of thiocetamide administration resulted, after a period of four days, in a decrease in nuclear volume though the increased polyploid values remained constant. From this evidence it seems that not only is nuclear enlargement following thiocetamide administration a result of increased ploidy but also, to some degree, due to increase in other constituents. The nucleolus could well be involved in this increase in size without increase in DNA content since one of the most conspicuous changes which ensues after thiocetamide involves enlargement of the nucleolus as well as

biochemical changes in this organelle (Fitzhugh and Nelson, 1948; Kleinfeld, 1957; Steele et al., 1965; Godwin et al., 1967). The mechanism by which the increased ploidy of nuclei is brought about has not been confirmed but it would not appear to involve mitotic inhibition since initiation of DNA synthesis and mitosis has been reported at doses seemingly below a necrotoxic level (Reddy, et al., 1969).

c) Ethionine

Histological evaluation of the hepatocyte population of the liver has shown that ethionine is capable of inducing the formation of enlarged cells with hyperchromatic nuclei within two weeks of inclusion of this compound in the diet of rats (Popper and Bruce, 1955; Farber, 1956).

d) N-2-acetylaminofluorene (AAF)

Farber (1956) noted the formation of enlarged liver cells with hyperchromatic nuclei in histological sections of rat liver after prolonged administration of AAF.

3. Evidence of nuclear damage

The mechanism by which radiation damage causes disturbances in the cell cycle has not been established but suggestions of direct nuclear damage have been put forward (Post and Hoffman, 1957; Puck and Marcus, 1956) and a similar postulate has been made with the pyrrolizidine alkaloids (Jago, 1969; McLean, 1970). It would seem therefore, to be appropriate to compare the hepatotoxins capable of inducing enlarged parenchymal cells from this standpoint.

Some of the hepatotoxic factors discussed above cause nuclear

damage which has been evaluated either by light and electron microscopy or by biochemical evidence of interaction with nuclear constituents.

Damage to chromosomal material resulting from hepatotoxic agents commonly manifests itself as abnormal mitotic figures which are observable with the light microscope. In this way radiation damage has been shown to produce marked mitotic abnormalities in the regenerating rat liver (Albert, 1958; Weinbren, et al., 1960; Leong, et al., 1961; Cuttis, et al., 1964). Much of this damage appears to persist in latent form in interphase nuclei for many months following the initial irradiation (Albert, 1958; Weinbren et al., 1960). Bombardment with x-rays of small areas of chromosomes in mitotic figures has produced marked abnormalities which became apparent as the mitoses proceeded (Zirkle and Bloom, 1953).

An increase in the incidence of chromosomal abnormalities at cell division, such as fragmentation, formation of bridges and spindle defects, were seen during prolonged DAB feeding (Stitch, 1960) and after necrotoxic doses of the pyrrolizidine alkaloid lasiocarpine (Schoental and Magee, 1959).

Increased rates of mutation are seen following irradiation with x-rays (Stromness and Kvellard, 1963; Brown, 1964; Newcombe and McGregor, 1965) and following exposure to pyrrolizidine alkaloids (Clerk, 1960; Avanzi, 1961; Alderson and Clerk, 1966) and Aflatoxins (Legator, 1969). The available evidence is consistent with the view that this enhanced mutation rate indicates increased chromosomal damage.

Ultrastructural evidence of nuclear damage was reported by Svoboda and Reddy (1968). In acute experiments, aflatoxin, DAB, lasiocarpine, thiocetamide, AAF and ethionine all brought about some degree of rearrangement of the fibrillar and granular elements of the nucleolus.

In chronic experiments, peripheralization or condensations of the fibrillar component of the nucleolus were present in livers of animals given DMN, aflatoxin B₁ and DAB. With thiocetamide marked increase in the granular component persisted while, with ethionine, nucleolar enlargement due to increase in both fibrils and granules was present. Interchromatin granules in the nucleus were increased with aflatoxin B₁, lasiocarpine and thiocetamide in acute experiments and with DMN and ethionine during chronic administration. It was suggested that such granules might represent sites of extranucleolar RNA synthesis. Apart from this, no specific significance has been attached to the nucleolar or nuclear changes observed in either acute or chronic experiments.

The chemical hepatotoxins which would appear to bring about nuclear enlargement through an inhibitive action on mitosis have been shown to interact with nuclear constituents. Aflatoxin (or its metabolites) have been shown to bind to liver DNA and RNA both in vitro (Clifford and Rees, 1965; Sporn et al., 1966) and in vivo (Lijinsky, 1968). Similarly, binding of metabolites of the amino-azo dyes with rat liver protein in vivo (Miller et al., 1949) and to liver RNA in vivo (Marroquin and Farber, 1963) has been demonstrated and Warwick (1967) and Warwick and Roberts (1967) have reported the binding of radioactive metabolites of DAB to rat liver DNA in the partially hepatectomised rat.

The pyrrolizidine alkaloid, lasiocarpine, has also been shown to possess weak alkylating potential with regard to nucleic-acids in vitro (Culvenor et al., 1962) and more recently the possibility has been suggested that this also occurs in vivo (Culvenor et al., 1969).

Of the remaining chemicals under discussion carbon tetrachloride has not as yet been shown to interact with nuclear constituents. In contrast to this, as will be seen in the next chapter of this review, DMN is a potent alkylating agent of nucleic acids and protein. In spite of the ultrastructural damage seen following thiocetamide administration, no biochemical evidence of damage to chromosomal material has been produced with this compound. Ethionine poisoning, however, has recently been shown to lead to the rapid ethylation of liver protein (Natori, 1963; RNA, (Farber et al., 1967; Rosen, 1968) and the guanidine moiety of DNA (Stekol, 1965; Swann et al., 1971). In the case of AAF, persistent binding of the compound to rat liver DNA, RNA and protein in vivo has been observed (Irving and Richard, 1969).

4. Necrotoxic and carcinogenic action

A major part of this work investigates the action of DMN on liver cell enlargement, and the possible association of this change with carcinogenesis. It will be seen in the next section of the literature review that DMN has been shown to be a powerful alkylating agent of DNA, RNA and protein both in vitro and in vivo. Furthermore, many workers favour the concept that this type of interaction could well be involved in the carcinogenic activity of the compound. With this aspect

in mind, the tumorigenic and necrotoxic potential in the liver of the hepatotoxic chemicals under consideration will now be discussed.

a) Necrosis

Carbon tetrachloride induces marked centrilobular necrosis in the rat liver but the carcinogenic activity of this chemical is questionable. As with carbon tetrachloride, most of the remaining hepatotoxic chemicals cause centrilobular necrosis in rat liver when administered at suitable high levels. Thus, DAB brings about centrilobular necrosis in the liver at dosage levels exceeding 250 mg/kg (Orr 1948). The acute lesion seen in rats dying within the first three to five days of a single 100 mg/kg dose of the pyrrolizidine alkaloid, retrorazine (Selzer et al., 1951) or from a single 1500 mg/kg dose of lasiocarpine (Schoental and Magee, 1957) is essentially one of centrilobular haemorrhagic necrosis. DMN produces a severe centrilobular necrotic lesion within twenty-four hours of administration of the LD₅₀ dose (Barnes and Magee, 1954). Finally, thiocetamide at levels around 100-200 mg/kg also induces a centrilobular necrosis (Ambrose et al., 1949; Gupta, 1956b) within twenty-four hours of administration.

The action of aflatoxin on rat liver differs from the above compounds in that it induces necrosis which has a periportal distribution (Butler, 1964). The location of this acute lesion in species other than the rat is of considerable interest since in the duckling and cat it is periportal, while it is midzonal in the pig, and centrilobular in the dog and guinea pig (Newberne and Butler, 1969). Haemorrhage, necrosis and massive accumulation of lipid has been reported in the livers of monkeys

exposed to aflatoxin B₁ (Rao and Gehring, 1970).

Wilson et al., (1941) were able to find little evidence of acute toxicity in the liver following administration of high levels of AAF to the rat while the administration of ethionine has shown lipid accumulation rather than necrosis of the liver to be the predominant lesion (Farber et al., 1950).

b) Carcinogenesis

Lancaster et al., (1961) reported a high incidence of hepatic carcinoma in the rat following the ingestion of a diet of ground-nut meal contaminated with the aflatoxins. Tumour induction has since been confirmed in the rat following prolonged feeding and in more limited exposures to aflatoxin (Butler and Barnes, 1963).

Tumours have also been induced by a single LD₅₀ dose (7.6 mg/kg body weight) administered to female rats, when the mean tumour induction time was approximately six months (Carnaghan, 1967). When considering the carcinogenic potential of aflatoxin it is of interest that a protein deficient diet has been reported to enhance the acute necrotoxic action in the rat (Madhavan and Gopalent, 1965; Goodall, 1968). Low protein diets decrease the level of some drug metabolizing enzymes of the liver (McLean and McLean, 1966) and on this basis it would seem that the acute necrotoxic effect is induced by the unchanged molecule. The carcinogenic effect of aflatoxin also appears to be affected by the unchanged molecule, since McLean and McLean (1969) reported that simultaneous phenobarbitone and aflatoxin administration to rats resulted in a decreased tumour incidence compared to appropriate

control animals. Phenobarbitone increases the level of some drug metabolizing enzymes in the liver (McLean and McLean, 1966).

Finally, aflatoxins have also been shown to be definitive hepatocarcinogens in the duckling (Carnaghan, 1965) and the trout (Halver, 1965). The possible connection of human liver neoplasia with ingestion of food contaminated with aflatoxins has been emphasized (Newberne and Butler, 1969; Butler, 1970).

Fischer (1906) reported that the sub-cutaneous injection of scarlet red into the ear of the rabbit caused a temporary hyperplasia of the epidermis. Being interested in the proliferative action of the azo-dyes, Yoshida (1932; 1933) studied the chronic effect of long term feeding of another azo-dye, orthoaminoazo-toluene, and succeeded in producing liver tumours. Kinoshita (1936) following on in this field of research reported that DAB was a much stronger hepatocarcinogen. Orr (1940) confirmed the production of liver cell carcinomata, bile duct carcinomata and bile duct cystadenomata after prolonged feeding of DAB incorporated in the diet.

In spite of a number of investigations carried out with various pyrrolizidine alkaloids the carcinogenetic potential of these compounds remains in doubt (McLean 1970). The first investigation into this aspect of their toxicity was carried out by Cook et al., (1950) in which two out of 17 rats receiving alkaloids of Senecio jacobaea intermittantly in their drinking water developed hepatomata and cystadenomata. The tumours arose approximately one year from the commencement of the experiment. This result could hardly be called



unequivocal and Schoental et al. (1954) carried out further experimentation in which longer survival of the animals was ensured. The rats were treated three days weekly with one or a mixture of alkaloids of S. jacobaea, retrorsine or retrorsine N-oxide. A trabecular hepatoma was found in the liver of one rat out of the 25 administered the mixture of alkaloids for one year. Of 14 rats administered retrorsine, four rats developed hepatomata. The 22 rats given retrorsine N-oxide were divided into three experimental series. Administration via the drinking water gave rise to one hepatocellular carcinoma with metastases, and nine hepatomas (10/22 incidence). In the third series five rats were painted with 0.5% solution of retrorsine N-oxide on the nape of the neck. These animals survived 11 to 18 months and the liver of one animal dying at the end of this period contained a hepatoma. Local skin lesions did not develop from this treatment. Bull et al., (1968) appraised the above publication in the light of their own experience and concluded that many of the hepatomata might well have been regenerative nodules of hyperplastic rather than neoplastic liver cells. Furthermore, as McLean (1970) stressed, the rats were maintained on a diet containing peanut meal and subsequent involvement of aflatoxin thus cannot be excluded.

Dybing and Erichsen (1959) fed male rats a diet containing 1% of the plant Senecio aquaticus. All of the animals died in 7-9 months and all livers showed gross distortion of architecture. These authors were uncertain whether to label nodules of parenchymal cells as hyperplastic or neoplastic.

Bull et al., (1968) emphasised that the criteria on which experimentalists had based a diagnosis of liver neoplasia following exposure to pyrrolizidine alkaloids leave considerable room for doubt as to their carcinogenic potential. These workers reported a number of their own small experiments in which pyrrolizidine alkaloid administration failed to induce neoplasia but in which regenerative nodules in the liver were a very prominent feature.

McLean (1970) enlarging on the carcinogenic potential of these alkaloids pointed out that liver neoplasia has not been reported following any of the many acute exposures of domestic animals to these alkaloids. Although a relevant point, the lack of control of many aspects of spontaneous domestic animal poisoning is not helpful in reaching any sure conclusions. This worker formed the opinion however that continued administration with a persistent antimitotic effect would be unlikely to allow the development of tumours. Cessation of treatment followed by life-span studies, so that regenerative colonies of hepatocytes are able to develop, might well give rise to neoplasia in the liver. McLean (1970) includes a table in her review which very clearly indicates that it has been the studies of pyrrolizidine alkaloids which have involved cessation of exposure and allowing the animals to live on, that have resulted in the eventual finding of liver tumours. Even so the incidence of liver tumours was always low in these experiments.

On the evidence of the work of Fitzhugh and Nelson (1948) and Gupta (1956a), thiocetamide appears to be weakly carcinogenic since continuous feeding resulted in liver cirrhosis and eventual tumour

formation.

Similarly, ethionine fed at low levels over a prolonged period produced hepatocellular tumours (Farber, 1956). This result was confirmed by Gelboin et al. (1958) and Sorof et al. (1958).

The carcinogenicity of the compound 2-acetylaminofluorene was demonstrated by Wilson et al., (1941) during the screening of potential insecticides and has since been confirmed (Skoryna and Webster, 1951; Reuber, 1968). Recently it has been suggested that AAF requires to be metabolised to N-hydroxy-2-acetylaminofluorene to realise its carcinogenic potential in the rat (Miller, et al., 1960; Miller et al., 1961; Weisberger et al., 1965).

Finally, x-irradiation of mice has induced liver tumours (Curtis et al., 1968). A possibly insignificant proportion of mice administered tritiated thymidine have also been reported to have developed liver tumours one year after administration (Lisco et al., 1961).

5. Conclusions

It is apparent from the evidence outlined above that there are at least two mechanisms by which pathological nuclear enlargement is brought about in the liver.

One of these systems is an extension of the physiological nuclear enlargement which was described in Chapter I. It would seem that a regenerative increase in mitosis induced in the liver by either chemical or physical means, initiates division of a proportion of the binucleate hepatocyte population. The division of these binucleate cells then

leads to the formation of a binucleate cell or two mononucleate cells which contain nuclei of the next ploidy level. If any other mechanism is involved it has not made itself apparent from the studies on the adult liver.

There are other hepatotoxins which, like carbon tetrachloride, are necrotoxic but with which the healing response is very different. In the case of these compounds, although a reparative wave of DNA synthesis is instigated, the entrance of the cells into mitosis is inhibited and nuclear ploidy increases as a consequence of this interruption of the cell cycle. Furthermore, in the specific instances of the pyrrolizidine alkaloids and radiation damage, nuclear damage induced by their action has been suggested as the basis of their interference with the cell cycle. If one also looks at the carcinogenic potential of the hepatotoxins in this group (See Table 2.1.) it may be seen that they have all been shown to be capable of inducing neoplasia in the liver.

Finally, there are those compounds mentioned in this chapter and shown in Table 2.1 which are also carcinogenic and invoke cell and nuclear enlargement, but cellular kinetics have not as yet been sufficiently studied to ascertain precise mechanisms.

From this present state of knowledge it was decided initially to attempt to differentiate between liver necrotoxins and potent carcinogens by studying cellular kinetics following single administration to the neonate rat. (The reasons for choice of the neonate rat in this work may be found briefly outlined on p 29 of the previous chapter). It was intended that particular attention would be paid to the mode of

formation of enlarged hepatocyte nuclei induced by hepatotoxins. At the commencement of the work the following three investigations were envisaged.

(1) Confirmation of the mechanism by which a compound which is primarily a necrotoxin induces nuclear enlargement in the liver. Carbon tetrachloride was chosen for this purpose.

(2) Investigation of the mechanism of nuclear enlargement induced with compounds in the last group of which little is known of the cellular kinetics following their administration. Dimethylnitrosamine, a potent carcinogen, was chosen primarily for this purpose, but a brief investigation was also carried out with thiocetamide.

(3) Investigation of the carcinogenic response of a single administration of DMN and carbon tetrachloride to the neonate rat. It was thought that such a dosing regimen would confirm the extreme carcinogenic potential of DMN and at the same time justify the classification of carbon tetrachloride as a primarily necrotoxic agent.

From these studies it was hoped to gain further knowledge on the nature of pathological nuclear enlargement and the association of any mechanism of enlargement with carcinogenesis.

CHAPTER 4

HEPATOTOXICITY OF DIMETHYLNITROSAMINE

AND CARBON TETRACHLORIDE

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A major part of the work of this thesis involved the use of dimethylnitrosamine and carbon tetrachloride; The following pages contain a more detailed review of the toxicity of these two compounds.

A. Dimethylnitrosamine

DMN is a nitroso compound and since these compounds are well known toxic agents consideration must be given here to the group as a whole. Thus reference is made to other compounds of this group where work on them appears to have a direct bearing on the action of dimethylnitrosamine itself.

The incrimination of dimethylnitrosamine as a hazard in industry, where its use as a solvent resulted in acute or sub-acute illness in workers, initiated laboratory investigations into its possible toxicity (Barnes and Magee, 1954). This early work showed dimethylnitrosamine to be a potent hepatonecrotxin in the rat, rabbit, mouse, guinea pig and dog. Later work by these authors (Magee and Barnes, 1956), demonstrated that prolonged administration of dimethylnitrosamine at low levels in the diet of rats resulted in the development of liver tumours within a year. From these experiments has stemmed a great deal of work on the toxic and carcinogenic activity of dimethylnitrosamine and other related compounds. As a result of this demonstrable carcinogenicity of DMN and many other nitroso- compounds, an association with the epidemiology of human neoplasia has been suggested. (Sen et al., 1959; Magee and Barnes, 1967; Magee, 1971).

1. Dose response

a) Single dosage

i) Lethality

The oral LD₅₀ for dimethylnitrosamine is given as between 27 and 41 mg/kg for adult rats (Heath and Magee, 1962) with death usually taking place within the first forty-eight hours following dosage.

ii) Histology

Above 20 mg/kg, DMN administered by any route to the adult rat produces a pronounced centrilobular necrosis of the liver (Barnes and Magee, 1954), while Hultin (1960) reported that below 15 mg/kg this effect is not produced. The repair of the lesion, noticeable by increased mitoses of parenchymal cells, is seen approximately forty-eight hours following administration and is complete three weeks after dosage (Barnes and Magee, 1954). This necrotoxic effect of dimethylnitrosamine has also been demonstrated in the liver of the Pekin duckling (Carl et al., 1966) and the Syrian hamster (Herrold, 1967). Barnes and Magee (1954) commented on the enlarged pale staining hepatocyte nuclei seen eighteen hours after the administration of a single dose. This effect on the enlargement of parenchymal cell nuclei has also been observed following administration of diethylnitrosamine to the rat (Grundman and Sieberg, 1962). A veno-occlusive lesion was reported ten days after a single 30 mg/kg dose of dimethylnitrosamine in the rat, (McLean et al., 1965; McLean et al., 1969) and a similar lesion was observed in mink exposed to the compound (Carter et al., 1969).

Experimental procedures can be carried out which markedly influence the necrotoxic response of the liver to dimethylnitrosamine. In this connection, protein depletion protects against the lethal and hepatotoxic effects in rats (McLean and McLean, 1969; McLean and Verschuuren, 1969). The likely mechanism of this protection against liver damage seems to be that protein deficiency depresses the activity of the enzyme system in the liver responsible for metabolising dimethylnitrosamine to a toxic alkylating agent (Magee and Swann, 1969). The administration of benzo-(a)-pyrene partially reverses this effect of protein depletion and indicates that this compound is capable of inducing the enzyme system that is responsible for metabolising DMN (McLean, unpublished).*

The observations made by light microscopy have been supplemented by studies with the electron microscope on the acute lesion. In the cytoplasm of the cell there is early detachment of ribosomes and dilatation of the cisternae of the endoplasmic reticulum within eight hours of injection of dimethylnitrosamine (Emmelot and Bennedetti, 1960). These observations have since been confirmed by Mukherjee et al. (1963) and have, as will be seen later, an important association with the effect of DMN on protein synthesis. Ultrastructural nucleolar changes have also been observed with the electron microscope, involving separation of constituents, a process termed microsegregation (Svoboda and Higginson, 1968).

* Quoted in McLean and Magee (1970)

iii) Carcinogenicity

A single sub-cutaneous injection administered to the neonate rat resulted in the induction of renal tumours and hepatomas after twelve months (Terracini and Magee, 1964). This work involved injecting 125 μ l DMN per rat into two groups of animals, one of which was composed of rats less than twenty-four hours old and the other in which the rats were seven days old. The tumours were recorded in both groups with anaplastic renal tumours predominating. More recently, Terracini and co-workers (1969 - personal communication) induced liver tumours in twenty out of thirty-four rats injected with 125 μ l DMN/rat at less than twenty-four hours of age. Twenty out of the thirty-four rats also developed renal tumours and a low incidence of mammary tumours was also seen. Magee and Barnes (1962) reported the appearance of kidney tumours in adult rats surviving a single LD₅₀ dose of dimethylnitrosamine. When protein deficient diets are fed to rats, a single dose of 60 mg DMN/kg body weight will induce tumours of the kidney in virtually 100% of the survivors of the treatment (Swann and Magee, 1968; Hard and Butler, 1970a). This is postulated to be due to the fact that protein depletion leads to decreased metabolism of DMN by the liver and the compound is thus present in the blood for a longer period than in normally fed rats. This in turn allows a more prolonged exposure of the kidney to DMN and an increased incidence of tumours in this organ thus ensues. (McLean and Magee, 1970).

Hepatomas and lung adenomas have been induced following single injections of DMN to the newborn mouse (Toth et al., 1964; Terracini, et al., 1966).

b) Multiple dosage

The studies on the pathological effects of prolonged administration of DMN have mostly been aimed at elucidating the nature of the carcinogenic response. This response has varied considerably from one dosing regimen to another. The carcinogenic effects of other N-nitroso compounds have attracted many studies (See review Magee and Barnes, 1967) but the following will concern only dimethylnitrosamine.

i) Lifespan studies

In the rat, Magee and Barnes (1956) showed that DMN fed at 30 ppm in the diet resulted in an almost 100 percent induction of hepatocellular tumours twenty-six to forty weeks from the commencement of administration. (≈ 4.5 mg DMN/week)*. The tumour incidence in two groups of rats kept on diets containing five and two ppm respectively showed that even at these very low concentrations induction of liver tumours was possible (Terracini et al., 1967).

DMN in the diet of the mouse was shown to induce liver tumours exclusively (Takayama and Oota, 1963), similar results being obtained in the rabbit (LePage and Christie, 1969) and in the trout (Halver et al., 1962; Ashley and Halver, 1968).

ii) Definitive time studies

Administration of DMN for shorter periods but at higher levels appears to alter the carcinogenic response. In this respect it has been reported that administration of the compound at a level of 600

*Personal calculation on amount of compound ingested assuming an average food intake of 20 gms per day for an adult rat approximately 150-300 gms in weight.

mg five times weekly by stomach tube for four to five weeks (total administered approximately 10 mg/kg)* produced a wider range of carcinogenic activity with tumours being observed in the liver, lungs and kidneys (Argus and Hoch-Ligeti, 1961). Tumours of mainly the kidney but also the liver and lungs were induced by feeding rats diets containing 50-2000 ppm for periods varying from one week to three months and then returning them to standard feeds (total ingested thus varied between 7.5 to 30 mg per week)* (Magee and Barnes, 1962). Daily injections of 8 mg DMN/kg body weight over six days gave rise eventually, to a high incidence of kidney tumours (Jasmin and Riopelle, 1968).

With regard to other species, hamsters when given DMN in the drinking water as a 0.0025% solution for eleven weeks showed a predominance of tumours of the liver (Tomatis et al., 1964). Weekly subcutaneous 1 mg and 0.5 mg injections to two groups of Syrian hamsters, for two to fourteen weeks and four to five months respectively, led to the induction of tumours of the liver and post-nasal cavity (Herrold 1967). DMN administered as a 0.001% solution in the drinking water of mice for 141 days resulted in a very wide spectrum of tumours arising between the fifteenth and thirty-ninth week following the commencement of dosing. A high incidence of lung adenomata was recorded together with haemangiomata in the liver and tumours of the ovary, spleen, lymph node, para-renal tissue and one tumour in a peripheral nerve. (Toth et al., 1964). A high incidence of liver vascular tumours, hepatomata and lung adenomata were also induced following a seven day exposure of mice to DMN in their drinking water (Terracini et al., 1966).

iii) Nature of tumours induced in the liver

In the rat liver, the type of tumour which develops following administration of dimethylnitrosamine is most often hepatocellular in origin (Magee and Barnes, 1967). They are mostly carcinomatous and have a trabecular or anaplastic appearance often with central necrosis. Also frequently found are transcoelomic and lung metastases (Magee and Barnes, 1956; Terracini et al., 1967). This is not to say that tumours of other components of the liver have not been reported in the rat since Terracini et al. (1967) observed spindle cell carcinomas, Magee and Barnes (1962) a tumour of intrahepatic bile ducts and Geil et al. (1968) a haemangioma, a bile duct tumour and a fibrosarcoma.

Liver tumours in the rabbit (LePage and Christie, 1969) and trout (Halver et al., 1962) following DMN administration would appear to be essentially of the same hepatocellular origin as those seen in rats. In hamsters, cholangiocarcinoma and hepatocellular tumours (Tomatis et al. 1964) and haemangiosarcomas have been observed (Herrold, 1967). Studies with mice have shown that the induction of predominantly vascular tumours is possible by prolonged feeding of the carcinogen (Toth et al., 1964). On the other hand, a seven day exposure of relatively high levels in drinking water resulted in a high incidence of hepatomata (Terracini et al., 1966).

It is clear from the work on the carcinogenicity of DMN which has been reviewed above that the organ site and nature of the tumours induced may vary from one species to another and differs according to the level and duration of exposure and the age of the animal. In the

adult rat, for example, it would seem that high levels of the compound administered for short periods results in predominantly kidney tumours with the occurrence of some hepatomata and lung adenomata while low level continuous administration induces mainly liver tumours.

Single injection to the neonate rat, however, results in a high incidence of both liver and kidney tumours.

As will be seen below this carcinogenic response is of interest when considered in relation to the metabolism and mechanism of actions of the compound.

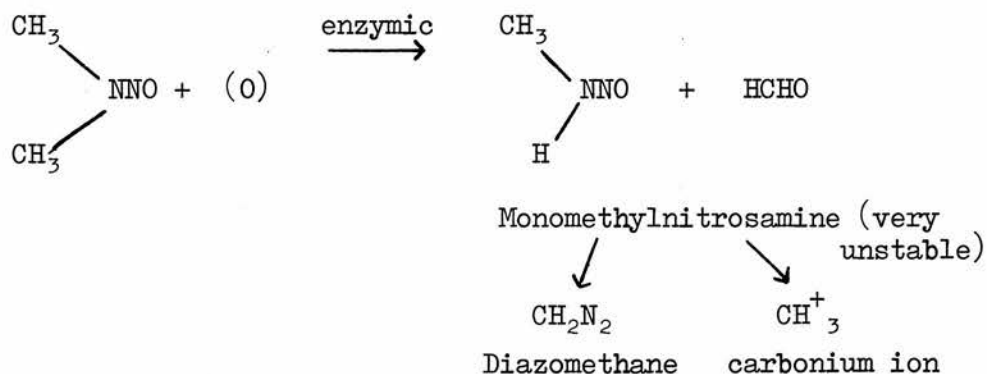
2. Metabolism

Experiments with rats, mice and rabbits have shown that, following administration, DMN is rapidly and evenly distributed throughout the body water (Magee, 1956). The recovery of the unchanged compound from tissues fell to zero within twenty-four hours of administration and since excretion in urine and faeces was slight it was concluded that DMN was rapidly metabolised. The main site of this metabolism is in the liver as is shown by the observation of Magee (1956) that the fall of DMN levels in tissues of the hepatectomised rat is very much slower than in the intact animal. A second experimental system confirmed this swift breakdown when Dutton and Heath (1956) demonstrated the rapid expiration of (^{14}C) carbon dioxide following the injection of (^{14}C) labelled DMN. In the newborn rat the disappearance of the compound over the first twelve hours is slower than in the adult rat but significant levels were again not detectable by twenty-four hours. (Terracini and Magee, 1964). In vitro experiments (Magee and Vandekar,

1958) have demonstrated the metabolism of DMN in rat liver slices and in microsome plus cell-sap preparations. Metabolism of the compound has also been reported in vitro in kidney slices but to a much lesser degree than in liver (Magee and Farber, 1962).

Dutton and Heath (1956) were the first workers to show that dimethylnitrosamine is demethylated in vivo. Later work (Mizrahi and Emmelot, 1962) which demonstrated the protective role of cysteine against DMN hepatotoxicity, is in agreement with the results of the former authors, in that cysteine inhibits demethylating enzymes in the liver. Heath and Dutton (1958) followed up their initial experiments by attempting to isolate likely active metabolites of DMN. Procedures carried out in this work for the identification of methylamine, hydrazine, nitrite and hydroxylamine and their conjugates were largely unsuccessful but traces of methylamine were found in liver and urine. From a comparative study of various nitrosamines Heath (1962) suggested that the acute effects of these compounds was due to an intermediate metabolite, possibly carbonium ions or diazoalkanes formed via an intermediate mono-alkylnitrosamine.

e.g. DMN (Magee, 1964)



With even distribution of DMN in the body water, and knowing that the main site of acute toxicity and metabolism is in the liver, it seems that a metabolite of some kind rather than the unchanged molecule must be responsible for the necrotoxic effects. Such a concept is further supported by evidence of a protective action of aminoacetonitrile against liver necrosis in the rat, the mechanism of which is believed to be by an inhibition of the metabolism of DMN (Fiume 1970).

3. Biochemical lesions

The inhibitory effect of dimethylnitrosamine on the incorporation of amino-acids into protein in vivo was reported by Magee (1958). Magee demonstrated that the incorporation of (^{14}C) amino-acids into liver protein was reduced by approximately 50% within three hours of a necrotising dose of DMN. This and other evidence on the incorporation of (P^{32}) into RNA suggested that this protein inhibition could be due to damage to microsomal structures. The inhibitive action on protein synthesis by DMN has also been demonstrated in vitro by Hultin *et al.* (1960) who with others (Brouwers and Emmelot, 1960) concluded that the impairment of protein synthesis involved a defect in the transfer and incorporation of the amino acids from sRNA¹ to the microsomal protein rather than at an earlier stage. Further, the electron microscope studies on Emmelot and Benedetti (1960) had shown, as previously mentioned, a disruption of ribosomes, and Mizrahi and Emmelot (1964) utilizing the polyuridylic acid saturation technique of sites of mRNA² on ribosomal particles, concluded that the specific effect was due to

¹ Soluble ribonucleic acid

² Messenger ribonucleic acid

loss of mRNA. This concept was supported by the work of Mager et al. (1965) who also used the polyuridylic acid technique and by the experiments of Villa-Trevino (1965; 1967) which demonstrated the methylation of purified RNA in the rat liver following administration of DMN. However, Magee and Swann (1969) pointed out that recent observations with the electron microscope by Benedetti and Emmelot (1966), in which damage to actual ribosomal particles was seen, indicate that factors in addition to loss of mRNA may be concerned in the inhibition of protein synthesis.

Evidence of interaction of the carcinogen with cellular constituents was provided by the work of Magee and Hultin (1962) in which the in vitro alkylation of protein and nucleic acids was demonstrated. The same work investigated the nature of this interaction in the intact rat when it was found that alkylation of liver and kidney protein and RNA, and liver DNA occurred. In the nucleic acids it was the guanidine moiety of the bases which was significantly methylated. Craddock and Magee (1963) later produced evidence of the methylation of kidney DNA. This study also investigated the variation of methylation with time following a single dose. Liver and kidney RNA alkylation reached a peak five to twelve hours after injection and then decreased reaching very low levels by three weeks. In the case of liver DNA the methylated base was lost more rapidly.

Methylation of RNA in the neonate rat has also been studied following DMN administration and was shown to increase in extent over the first three days of life after which adult levels were reached

(Lee and Spencer, 1964). Moreover, the level of alkylation in the kidney was found to be higher than in the liver over the first three days following birth which is interesting when viewed in the light of the high incidence of kidney tumours following single administration of DMN to the newborn rat.

Other studies on the acute biochemical effects of dimethylnitrosamine in the rat have shown an early reduction in the level of liver RNA (Magee, 1958) loss of glycogen (Emmelot and Benedetti, 1960), increased lipid (Rees and Shotlander, 1963) and disruption of mitochondrial enzymes (Bailie and Christie, 1959).

4. Mechanism of action

The particular observations concerning the alkylation of DNA has prompted the suggestion that the carcinogenic effect of DMN is exerted by direct action of the compound on DNA (Magee and Farber, 1962; Lee and Spencer, 1964; Magee and Barnes, 1967; Lee and Goodall, 1968). In support of this, other carcinogenic N-nitroso compounds have been shown to alkylate guanine (Magee and Lee, 1963; Swann et al., 1965; Lee and Lijinsky, 1966) and a reasonable correlation appears to exist between the site of tumour formation and the degree of methylation of nucleic acids by N-nitroso and other alkylating agents in any particular tissue (Swann and Magee, 1968). That dimethylnitrosamine (and other nitroso-compounds, see review Magee and Barnes 1967) can have a profound effect on genetic coding is born out by its mutagenic action in Drosophila (Pasternak 1962). The indication is that this action might be due to the alkylating potential of DMN, as the compound is

non-mutagenic to Escherichia coli in which bacterium demethylating enzymes are absent. (Gessler, 1962).

Some facts do not seem to fit into this general concept of carcinogenesis by DMN however. Specifically Krüger et al. (1968) have claimed that N-ethyl-n-nitrosurea does not alkylate RNA in vivo even though it is both toxic and carcinogenic. Lijinski and Ross (1969) on similar grounds have also reported results which suggest that in vivo alkylation of nucleic acids by cyclic-N-nitrosamines might be unrelated to carcinogenesis by these compounds.

It is therefore appropriate at this point to finally mention an alternative hypothesis which has been advanced. (Arcos and Arcos, 1962; Argus et al. 1961). These authors suggested that since dimethylnitrosamine and other unrelated carcinogens are agents of protein denaturation then the action of the unchanged molecule might be more relevant than hitherto supposed. This hypothesis has been disputed by Magee and Barnes (1967) who laid stress on the fact that dimethylnitrosamine is rapidly and evenly distributed throughout the body water and yet the induction of neoplasia is tissue specific. Unless any particular tissue cell was more susceptible with regard to denaturation of cellular protein, the argument that DMN relies on this type of reaction for its carcinogenic expression holds little credibility.

B. Carbon tetrachloride

Apart from its use as an early anaesthetic, carbon tetrachloride aroused particular attention in the medical profession when Hall (1921) demonstrated the efficacy of this compound against hookworms in dogs.

In the wake of this discovery the use of carbon tetrachloride as an anthelmintic in humans initiated research into its possible toxicity.

Early experiments in the dog (Meyer and Pessoa, 1923) demonstrated the necrotoxic action of the compound on the liver. In the following years it was shown that this centrilobular necrosis seen in the dog could also be demonstrated in the guinea pig (Phelps and Hu, 1924), the rabbit (Hall and Shillinger, 1923), the rat (Lacquet, 1932) and the cat (Chandler and Chopra, 1925). The therapeutic use of carbon tetrachloride (Phelps and Hu, 1924) revealed the production of a similar lesion in the liver of man. While the poison has a wide toxic spectrum in mammals it is of interest that birds have been shown to be resistant to its liver damaging effects (Hall and Shillinger, 1923).

1. Dose response

a) Single administration

As indicated above, when administered at an adequately high level carbon tetrachloride has a marked necrotoxic effect on the centrilobular zone of the liver lobule; this is followed, under normal circumstances, by rapid regeneration from those cells which survive the initial onslaught.

Using rats, Cameron and Karuraratne (1936) demonstrated a dosage threshold for carbon tetrachloride necrotoxicity as evaluated by light microscopy and also showed that above this level increasing doses resulted in the further extension of the centrilobular zone of necrosis in the liver. This effect has since been shown to be modified considerably

by the protein content of the diet. McLean and McLean (1969) observed the virtual abolition of the lethal and hepatotoxic effects in rats maintained on a protein free diet. Simultaneous administration of compounds, such as phenobarbitone or DDT*, which stimulate microsomal enzymes, result in an enhancement of necrotoxicity (McLean and McLean, 1969; Seawright and McLean, 1967; Garner and McLean, 1969). These observations are of importance in the relationship between toxicity and metabolism of the compound, as will be seen later. The toxicity of carbon tetrachloride also varies with age as the neonate rat liver has been reported to be relatively resistant to the necrotoxic effects (Cameron and Karuraratne, 1936; Dawkins, 1963).

Histology

Reports of the changes in the liver seen by light microscopy following carbon tetrachloride administration are numerous e.g. (Lacquet, 1932; Cameron and Karuraratne, 1936; Christie and Judah, 1954; Post et al., 1960; Wigglesworth, 1964) and all are in agreement that the hepatocyte lesions of necrosis and hydropic degeneration develop between the fifth and twenty-fourth hours following administration. By the end of this period, the fatty change in the remaining zones of the lobule is seen to be advanced; repair in the adult rat is well underway by forty-eight hours and is complete within two weeks. Post et al. (1960) reported that regeneration in the young rat (the earliest studied was a group of three week old animals) was very much more rapid than in the adult.

* Dichloro-diphenyl-trichlorethane

Of the groups of workers cited above, the study of Wigglesworth (1964) deserves particular attention. Utilising an ethyl-osmium gallate technique this experimentalist was able to demonstrate by light microscopy, changes in the mitochondria of hepatocytes as early as one hour after dosing and the formation of lipid droplets within two hours. Overt necrosis was seen at twelve hours and this region was separated from portal areas by cells undergoing hydropic degeneration. The necrosis of hepatocytes had reached maximum levels by twenty-four hours.

Wigglesworth's observations of the effect of carbon tetrachloride on mitochondria are well substantiated by observations with the electron microscope. However, this ultrastructural method of observation has placed the earliest morphological defect in the endoplasmic reticulum. Reynolds (1963) reported a disintegration of the rough endoplasmic reticulum within fifteen minutes of oral administration of carbon tetrachloride and the earliest change in mitochondria at thirty minutes. There is general agreement between these observations and those of other workers in that the first change observed by electron microscopy involves a degranulation of the endoplasmic reticulum followed by mitochondrial swelling, both of these affects occurring within four hours of administration (Dianzani, 1954; Oberling and Rouiller, 1956; Bassi, 1960; Smuckler et al., 1962; Ashworth et al., 1963).

6. Multiple dosage

Repeated administration of carbon tetrachloride has been shown to produce cirrhosis of the liver and, in some instances, neoplasia.

i) Fibrogenesis

Given precise conditions, the repeated administration of carbon tetrachloride manifests itself in distortion of the architecture of the liver, the main feature of which is a massive increase in fibrogenesis. The relevance of increased fibrinogenesis to the pathogenesis of cirrhosis in man has been a point of keen interest in the toxicity of carbon tetrachloride. The origin of the fibrous tissue formed in cirrhosis is uncertain. On the one hand, it is thought that fibrosis occurs first in the centrilobular necrotic lesion, while, on the other hand, it is argued that fibrosis is originally portal in location and therefore, only indirectly related to the necrosis caused by carbon tetrachloride.

Specifically, the production of cirrhosis of the liver by carbon tetrachloride was shown by Cameron and Karuraratne (1936) to require doses large enough to cause necrosis and to be repeated at intervals of too short a duration to allow complete regeneration of the liver. When these conditions were fulfilled fibrosis and bile duct proliferation readily occurred. If the dosing is continued for long enough then this fibrogenic response is self-perpetuating (Aterman, 1954). Cameron and Kararatne (1936) proposed that the apparent early centrilobular fibrosis observed was a result of collapse of the liver architecture with condensation of reticular elements due to loss of hepatocytes in this area. These workers suggested that actual fibroplasia with formation of new collagen fibres originated in the periportal region. Other experimentalists have supported this concept of

carbon tetrachloride induced cirrhosis (Lacquet, 1932; Rubin et al., 1963). Cirrhosis of the liver induced by dietary factors (Glynn et al., 1948) other hepatonecrottoxins (Hulterer et al., 1961; Popper et al., 1961) and bile duct ligation (Hulterer et al., 1961) has also been taken to support the view that the pathogenesis of cirrhosis is essentially periportal in location.

Other authors (Moon, 1934; Ashburn et al., 1947; Aterman, 1954) have, however, postulated that the centrilobular region is the predominant site of fibrosis. This association of necrosis and fibrosis with the centrilobular region was observed by Ashburn et al. (1947) who marked the portal vessels of the liver by injecting them with charcoal in gelatine. It was concluded that it was the hepatic veins which were primarily involved in the fibrogenic response.

A number of factors have been shown to modify the development of cirrhosis in the rat, viz: diet, low protein exerting a protective role (Bhuyan et al., 1965), sex, the males being more susceptible after weaning (Reddy et al., 1962; György et al., 1946); cortisone administration producing inhibition (Aterman, 1954) and phenobarbitone dosage markedly potentiating the fibrogenic response to the compound (Seawright et al., 1968).

The observations on the liver by light microscopy following prolonged poisoning by carbon tetrachloride have been extended by electron microscopy. Two types of hepatocyte change have been recognised, the first involving what was considered to be extensive proliferation of the rough endoplasmic reticulum (R.E.R.) and the

second a lipid laden cell with reduced and dilated R.E.R. (Stenger, 1963; 1966). Both cell types contained increased numbers of polymorphic lysosomes. This author suggested that the cell type with increased R.E.R. was indicative of their adaptation towards an increased ability to detoxify carbon tetrachloride.

ii) Carcinogenesis

Tumours of the liver of the rat have been reported following prolonged carbon tetrachloride administration. Costa et al., (1963) described carcinoma of the liver after inhalation of the chemical but their work was criticised by other experimentalists who were inclined to regard the lesions possibly as hyperplastic nodules (Reuber and Glover, 1967). These latter workers reported hyperplastic nodules and hepatocellular carcinomata in the livers of rats given twice weekly necrotoxic injections of carbon tetrachloride for twelve weeks. These tumours were small (below 5 mm in diameter) and were designated as neoplastic on the atypical appearance of the hepatocytes and liver architecture. No metastases of these lesions were observed and animals were not allowed to live on past the twelve week period in order to study the further development of these lesions. No attempts were made to transplant these tumours. No other claims as to the induction of neoplasia following carbon tetrachloride administration in the rat have been made. Other reports following repeated injections (Cameron and Karuraratne, 1936) or gastric intubation (Shubick and Hartwell, 1957) reported failure to induce a neoplastic response.

There are early reports of tumour induction in the liver of the

mouse following prolonged administration of carbon tetrachloride (Edwards, 1941; Edwards et al., 1942; Eschenbrenner, 1944; Eschenbrenner and Miller, 1946). These studies claimed the induction of high incidences of hepatomata.

Della-Porta et al., (1961) investigated the carcinogenic effect of carbon tetrachloride in the hamster. Prolonged administration induced liver cell carcinomata, one of which metastasised, but attempts to transplant these tumours were unsuccessful.

2. Metabolism

Following oral dosing (Recknagel and Litteria, 1960) or subcutaneous injection (Dawkins, 1963) in the adult rat, the concentration of the chemical in the liver was found to rise rapidly to maximum levels within four hours of administration but to fall to low levels by twenty-four hours. In the newborn rat accumulation of the compound in the liver was more rapid (Dawkins, 1963). All these authors were in agreement that the maximum levels in the blood occurred within one hour of administration and declined slowly thereafter. Dawkins (1963) found that the elimination of carbon tetrachloride was more rapid in the newborn than the adult and he was inclined to attribute this difference to the low lipid reserves of the animal at this early age. That significant concentration of carbon tetrachloride does occur in fat depots has been demonstrated by MacCollister et al. (1950). In this work it was shown that inhalation of (^{14}C) carbon tetrachloride by monkeys for approximately two hours resulted in a concentration of radioactivity in the body fat

which was twice as high as that found in the liver. Other tissues studied showed relatively little activity. The metabolism of this compound was also demonstrated in this work when it was found that ten to twenty percent of the radioactivity in blood and expired air was present as (^{14}C) carbon dioxide. The other principal form was as a conjugate in urine. Further evidence pointing to the metabolism of the compound was produced by Butler (1961) who identified small amounts of chloroform in the expired air of dogs administered carbon tetrachloride. Butler also showed the chloroform was produced in a medium containing carbon tetrachloride when this was incubated with mouse liver slices. More recently McLean and McLean (1966; 1969) have shown that the liver injury produced by carbon tetrachloride seems to be completely dependent on its metabolism by microsomal hydroxylation enzymes. Concerning the distribution of the compound in the liver itself, Roque and Fedarka (1961) found that the highest proportion of radioactivity following injection of (^{14}C) carbon tetrachloride to the mouse was in the mid-zonal and centrilobular zones of the liver lobule at eighteen hours. Radioactivity remained at relatively high levels in these regions for at least forty-eight hours.

3. Biochemical lesions

The pathological facets of carbon tetrachloride fatty change and necrosis in the liver have evoked tremendous amounts of work on the biochemical changes which might underly the cellular disturbances which lead to these effects. Within the scope of this review it is possible only to outline the main aspects of this work.

One of the earliest hypotheses dealing with fatty change incriminated mitochondrial damage as the initiating subcellular lesion. As early as 1936, Cameron and Karuraratne had commented on possible mitochondrial involvement. Christie and Judah (1954), with the use of the electron microscope and biochemical methods, described the more precise nature of the change in these organelles as a swelling with leakage of soluble co-factors. At approximately the same time, Dianzani (1954) showed an uncoupling of oxidative phosphorylation in fatty liver. Disruption of the carboxylic acid cycle with consequent failure of fatty acid oxidation was thus offered as an explanation of lipid accumulation in the liver following poisoning by carbon tetrachloride. The swelling of mitochondria as observed above was shown to be due to the increased permeability of their membranes (Recknagel and Malamed, 1958) with subsequent release of many constituents, such as cytochrome c (Dianzani and Viti, 1955) pyridine nucleotides (Dianzani, 1955; Kasbekar and Sreenivasan 1956; Recknagel and Anthony, 1959), Vitamin B₁₂ (Kasbekar et al., 1959) and thiamine diphosphate (Dianzani and Dianzani Mor, 1957). This leakage was shown to result in a profound loss of respiratory control at the cellular level both in vivo (Dianzani, 1954; Calvert and Brody, 1958; Recknagel and Anthony, 1959; Recknagel and Lombardi, 1961) and in vitro (Artizzu et al., 1963; Calvert and Brody, 1958; Recknagel and Anthony, 1959).

Among this last list of authors, however, were numbered some who, on the basis of the time sequence of subcellular events, questioned the postulate that mitochondrial damage was the initial lesion causing

fatty change (Calvert and Brody, 1958; Recknagel and Anthony, 1959). Oberling and Rouiller (1956) demonstrated with the electron microscope that, as early as one hour following administration, significant damage had occurred in the endoplasmic reticulum but that damage to mitochondria was not discernable at this time. Moreover, biochemical evidence of lipid accumulation in the liver showed that there was a significant increase within one hour of administration of the poison (Schotz and Recknagel, 1960; Rees and Shotlander, 1963; Lombardi and Ugazio, 1965) but that biochemical markers of mitochondrial damage were not seen earlier than four hours following administration (Calvert and Brody, 1958; Recknagel and Anthony, 1959; Share and Recknagel, 1959). Thus, it became clear that mitochondrial damage could not be the initiating lesion of lipid accumulation in the liver following carbon tetrachloride administration, though the possibility remained that such damage could contribute to increased lipid at a later stage; the involvement of these respiratory organelles in the process of necrosis likewise remains an open question.

With the abandonment of the mitochondrial hypothesis experimentalists searched in other directions for an explanation of fatty change. In this connection Leduc and Wilson (1958) suggested that the early interference with the endoplasmic reticulum was the underlying cause of this change. Earlier work had shown that the liver secreted large amounts of triglyceride into the plasma as a lipo-protein complex (see review Recknagel, 1967) and Recknagel et al. (1960) proposed that the lipid accumulation was due to a failure of secretion of

triglycerides from the liver, a postulate supported by the fact that carbon tetrachloride could considerably decrease a post-Triton induced lipaemia (Recknagel, 1960). The major problem at this stage was that of finding out whether the inhibition of secretion of lipoprotein was due to a defect in the formation of protein in the liver or a failure of coupling of lipid to this protein to form the transportable lipo-protein. There was soon clear evidence that carbon tetrachloride caused a very early depression in protein synthesis (Seakin and Robinson, 1963; Rees and Shotlander, 1963) and the former authors proposed that this was the primary event leading to lipid accumulation. They had support for this view from the results of earlier work which had shown that puromycin, an inhibitor of protein synthesis, could also cause fatty liver (Seakin and Robinson, 1962). The validity of this relationship has been questioned, however, (Recknagel, 1967) since ethionine had been shown to cause fatty liver by an inhibition of protein synthesis but with a more delayed onset when compared to the effect produced by carbon tetrachloride (Lombardi and Recknagel, 1962). Furthermore, actinomycin D fails to induce fatty change even though it markedly inhibits protein synthesis and therefore suggested that the primary effect was most likely to be a defect in the coupling process of protein to lipid.

The mitochondrial damage and interference with protein synthesis as outlined above incriminated carbon tetrachloride in an intrahepatic cellular poisoning of triglyceride metabolism or secretion. A third hypothesis needs to be briefly mentioned which maintained that carbon

tetrachloride exerted its effect through massive catecholamine discharge.

Brody and Calvert (1960) and Brody et al. (1961) had demonstrated that carbon tetrachloride poisoning caused a considerable release of catecholamines from the adrenal medulla and, moreover, that adrenalectomy afforded marked protection against lipid increases and to a lesser degree, necrosis in the carbon tetrachloride poisoned liver. In addition, it was seen in this work that cord-sectioning protected against both of these effects. It was thus suggested that catecholamines (released on central nervous stimulation of the adrenal medulla) exerted their effects by diminishing hepatic flow with associated centrilobular hypoxia leading on to overt necrosis. The fatty liver was suggested to arise from enhanced mobilisation of fatty acids from peripheral stores. In connection with this hypothesis re-necrosis by carbon tetrachloride it should be mentioned that earlier workers (Glynn et al., 1948) had conceived of carbon tetrachloride acting directly on periportal hepatic cells causing swelling and mechanical obstruction of sinusoidal blood flow and thus bringing about centrilobular hypoxia.

The role of catecholamines in hepatotoxicity due to carbon tetrachloride have since been shown to be irrelevant to the problem of the mechanism of action. Very briefly, in the case of necrosis it was shown that the protection seen in cord-sectioned rats was due to the profound hypothermia produced in the animal by this treatment, with a consequent lowering of the rate of metabolism in the liver (Larson and Plaa, 1963; Larson et al., 1964; Larson and Plaa, 1965). Furthermore, administration of large doses of catecholamines to intact rats failed

to induce any pathological effect resembling that seen after carbon tetrachloride administration (Larson and Plaa, 1965). The postulate that lipid accumulation is due to an over supply of fatty acids from lipid depots following catecholamine release is by no means supported by experiments showing that the elevation of plasma lipids is too little and too late to account for the increase in liver triglycerides by this means (Rees and Shotlander, 1963; Stern et al., 1965).

4. Mechanism of action

The exact mechanism by which carbon tetrachloride damages cellular organelles remains unknown, but following the suggestion of Butler (1961) and Recknagel and Ghoshal (1966) that it depended on the cleavage of the molecule with release of the free radical, the lipoperoxidation of cell membrane structural components now holds a dominant position as an explanation of the toxic action of the compound. In support of this, it has been shown that antioxidants protect against carbon tetrachloride necrotoxicity (Hore, 1948; Gallagher, 1961). More recently Fowler (1969) was able to isolate hexachloroethane from tissues of rabbits which had been administered carbon tetrachloride and concluded that this could well have arisen from dimerization of free trichloromethyl radicals.

C. Conclusions

On the basis of the above survey it is clear that both dimethyl-nitrosamine and carbon tetrachloride can be administered at levels which are sufficient to bring about centrilobular necrosis of the liver. Evidence to date suggests that it is an active metabolite of

each of these compounds which exert this effect. Present knowledge clearly indicates that DMN requires metabolism for the expression of its carcinogenic potential. In this respect DMN shows potent activity whilst carbon tetrachloride is at most only weakly active in this direction.

Both of these compounds have been shown to induce nuclear enlargement in the liver. Their choice as hepatotoxins in much of the experimental work of this thesis was influenced by the expectancy that the two substances would reflect their differential carcinogenic activity in the mechanism and nature of the nuclear enlargement which they induce in the parenchymal cell population.

S E C T I O N I I

T E C H N I Q U E S

CHAPTER 5

MATERIALS AND METHODS

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1. Miscellaneous

i) Animals

a) Sources of rats

The rats used in the experimental work of this thesis were from two sources viz:-

Carworth/CFHB strain - Wistar origin

(Carworth Europe, Huntingdon, England)

Ash/Wistar

(Scientific Products Farm, Ash, Canterbury, Kent)

The animals were housed in polythene and stainless steel cages and allowed free access to tap-water and pelleted diet (Oxoid pasteurised breeding diet, Oxoid London).

b) Breeding technique

This work is mainly concerned with studies of the livers of very young rats and so a nucleus of approximately forty breeding females was kept to maintain a constant supply of young animals.

These rats were mated in batches of four females to one male and the latter removed after a period of seven days. The females were separated into individual cages seventeen days after this and thereafter checked twice daily for litter births. The age of any litter was thus known to within approximately twelve hours.

Ages ascribed to animals in subsequent experiments are on the basis of the day of birth being day nought of life.

The neonate rats were sexed according to the anal-genital distance.

c) Identification

Since the suckling animals of any litter could not be separated and housed in respective treatment groups, it became essential to develop an efficient marking system. The most effective method was found to be by small subcutaneous deposition of tattooing ink using a small hypodermic needle. With one to four spots marked at the base of the tail, middle of the back or neck regions, individual animals could be identified as numbers one to twelve within litters. This method was used for experiments using animals up to three weeks of age after which the markings were obscured by the growing hair.

Litters used for long-term carcinogenic or autoradiographic experiments were marked by conventional ear or toe methods.

d) Methods of destruction

Animals less than two weeks of age were killed by decapitation.

Weanling animals were first stunned by a blow on the head and then exsanguinated.

Adult animals used in carcinogenic studies etc. were killed by ether inhalation.

ii) Chemicals used as hepatotoxins

Dimethylnitrosamine (N-nitrosodimethylamine) was obtained from Kodak Ltd., Kirby, Liverpool. This chemical was used throughout as a 300 mg % solution in physiological saline (0.85% NaCl).

Carbon tetrachloride ('Analar') was obtained from British Drug Houses and used as supplied.

Thiocetamide was also obtained from British Drug Houses (Poole England) and used as a 3,000 mg % aqueous solution.

iii) Administration

All chemicals were injected into the peritoneal cavity using a 250 μ l gas-tight syringe (Hamilton Co. Whittier, California) for dimethylnitrosamine and thiocetamide solutions, and a 10 μ l syringe (Hamilton Co. Inc.) for carbon tetrachloride.

The maximum dosing errors were calculated to be:

- (a) dimethylnitrosamine; \pm 2 mg/kg at a dose level of 10 mg/kg.
- (b) carbon tetrachloride; \pm 0.02 ml at a dose level of 0.1 ml/kg.
- (c) thiocetamide; \pm 10 mg/kg at a dose level of 100 mg/kg.

[N.B. Histological techniques used throughout this work may be found in the Appendix Section B together with a description of the method of sampling of portions of liver for quantitative experimental work].

2. Mitotic counts

As has been previously described, the liver of the rat increases rapidly in size during the first four to five weeks of life when compared to the growth rate of this organ in older animals. Even so the mitotic activity is still relatively slight, McKellar (1949) finding a lobular index of only 1 and Nadal and Zajdela (1966) a mitotic index of only 0.021 in the liver of the seven-day old rat. The counting of hepatocyte mitoses in the liver can therefore be a very tedious task if enough mitotic figures are to be scored to allow statistically valid conclusions to be drawn. For example, in their work on adult rat liver, Bass and Dunn (1957) found it necessary to count ten thousand hepatocytes in each liver sample.

For this reason it was thought that the use of a mitotic arresting agent would be advantageous so that cumulative counts could be made. Colchicine, at a dose level of 1 mg/kg, has been used extensively in the past for this purpose (Leblond and Walker, 1956) as well as the synthetic analogue colcemid, which is approximately ten times more active than colchicine itself (Leblond, 1959).

Ideally the properties which are required of a mitotic blocking agent are that:

- (a) the compound should be consistent in its action and arrest all cells entering mitosis.
- (b) it should not itself affect the rate at which cells enter division.
- (c) and lastly it should not be so toxic as to lead to death of cells within the time it is allowed to act.

Colchicine, and presumably colcemid, meet none of these criteria since the onset of mitosis may be inhibited in a certain percentage of the cells (Bureau and Vilter, 1939; Ludford, 1945). Further, Henry et al. (1952) found high doses of colcemid led to a relative decrease in the number of oral epithelial cells entering prophase. Another disadvantage is that at too high a dosage level, disintegration of dividing cells takes place, whilst at too low a level many cells are able to pass through the metaphase arrest. Thus the system is essentially a compromise to achieve the maximum arresting effect whilst the rate of entrance of cells into division and viability of those cells blocked, is not affected (Henry et al., 1952). It was with these factors in mind that Leblond (1959) recommended that the dose response of the agent should be evaluated on the particular tissue under test with regard to its mitotic blocking potential.

This was deemed to be of particular importance in the work presented in this thesis as it seemed unwise to extrapolate from suitable dose levels in the adult rat to the neonate animal where the metabolic activity (Jondorf et al., 1958) and the structural constituents (Dawkins, 1959) of the liver are still in the developmental stage.

For these reasons the mitotic blocking action of colcemid was first titrated in the liver of the two day old rat so as to establish a suitable dose for use in later experiments. This experiment is described at the end of this sub-section.

Materials

a) Colcemid

Obtained from Ciba Ltd. and used as a 10 mg% solution in 0.85% sodium chloride. The solution was stored in the dark at -4°C.

b) Fixative: Carnoys fluid

Absolute alcohol 60%

Chloroform 30%

Glacial acetic acid 10%

Method

In order to avoid variation due to circadian rhythms the animals were always dosed with colcemid at 10 a.m. and killed three hours later.

After the animals had been killed, a 3 mm thick slice of liver was taken in a transverse section across the liver and fixed immediately in Carnoys fluid. The tissue blocks from neonate livers contained representative portions of most of the lobes of the liver. In the case of the weanling animals only the medial lobe of the liver was utilised. The tissue blocks were left to fix for one and a half hours and then stored in 70% ethyl alcohol. As soon as practical the blocks were processed by routine methods for final embedding in paraffin wax. Sections were cut at 4 μ and stained with Erlich's alum haematoxylin and eosin.

A total of three thousand hepatocyte nuclei were scored in each liver section using a x 100 oil immersion objective and the numbers of mitotic figures in metaphase arrest recorded. A note was also taken of the anaphase-telophase mitotic figures as a check of adequate colcemid treatment, but these were not included in the final analyses. In all

the counts made, these late stages of mitoses never exceeded more than three per three thousand and it was more common to find none or only one such figure which had passed through metaphase.

The counting technique involved scanning the tissue section by moving one high-power field at a time. When the edge of the section was reached the stage of the microscope was moved four high-power fields at a right-angle to the original direction. The scanning and counting was then continued in the opposite direction to that originally taken. In the case of the neonate liver where more than one lobe was selected for histology the scanning direction was reversed when the final edge of the last of the liver sections was encountered. Only those interphase nuclei or mitotic figures, which were not partially excluded by the edge of the microscope field, were included in the counts.

Haemopoietic tissue is abundant in rat liver for at least the first week of post-natal life: this made the counting of mitotic figures more difficult than at later ages because of possible mistakes in the identification of cell types. Any mitotic figure to which any doubt at all was attached with regard to the cell of origin was recorded in a separate category and not included in the final analyses. The highest number of such mitotic figures found in any liver count was seven per three thousand though the more usual number lay between nought and three.

Establishment of an effective dose of colcemid
as a mitotic arresting agent in neonate liver

Materials and Methods

Two-day old rats were ascribed to one of five groups each of which consisted of six males and six females. Four groups were injected intraperitoneally with 0.02, 0.1, 0.2 and 1 mg colcemid/kg body weight respectively while a control group received 20 μ l physiological saline by the same route. The animals were dosed at 10 a.m. and killed three hours later, the livers then being processed, sectioned and appraised as described above.

Results

Table 1 in the Appendix shows the actual differential counts of mitotic figures observed with each liver section. Analysis of these results allowed the following conclusions. (In all statistical analyses in this thesis, unless otherwise stated, the square roots of observations were taken as a basis for the calculations to allow for any large discrepancies between the variances of groups).

1) An analysis of variance showed that there was no significant difference between groups with regard to the number of hepatocytes in prophase.

2) An analysis of variance on the counts of metaphase figures demonstrated a significant difference between groups.

$$F = 29.8 \quad n_1 = 4, \quad n_2 = 56 \quad p < 0.001$$

Inspection of the means of the groups and their respective standard errors gave the following result.

Treatment colcemid mg/kg	Control	0.02	0.1	1.0	0.2
Ranked means (sq.roots)	1.15	2.49	3.61	3.61	3.96
Standard error of means	± 0.208	± 0.216	± 0.216	± 0.216	± 0.216
p < 0.05	_____	_____	_____	_____	_____

Any two means not underscored by the same line are significantly different at $p < 0.05$.

3) Student 't' tests on each of the groups failed to reveal any significant difference between sexes.

4) When considering the number of hepatocytes blocked in metaphase and expressing this as a proportion of the total of metaphase, anaphase and telophase mitotic figures the following percentage arrest for each group was observed.

Control	71%
0.02 mg/kg	85%
0.1 mg/kg	93%
0.2 mg/kg	95%
1.0 mg/kg	97%

Other findings

One rat from the groups administered the top dose of 1.0 mg/kg was found to be in a comatose condition at the time of sacrifice.

No abnormalities were observed on macroscopic examination of the animals.

Histological examination of the livers of all animals failed to reveal any evidence of necrosis. One feature worthy of note however, was that the top dose level of colcemid induced considerable shortening of chromosomes so that mitotic figures were very much condensed as compared to mitotic figures seen at the lower dosage levels.

Conclusions

The similar incidence of prophase mitotic figures observed in all

the dosage groups and the control groups showed that the colcemid treatment did not per se stimulate cells to enter mitosis or inhibit them from so doing.

The relatively low counts of hepatocytes in metaphase and percentage arrest of all mitoses at a level of 0.02 mg colcemid/kg body weight indicated that this was not a suitable dose for subsequent use.

The top dosage level was also precluded from use in this work by the development of a comatose animal within three hours of treatment and by the extreme condensation of chromosomes at this level.

Of the remaining two groups, the 0.2 mg/kg level was chosen for subsequent use, it giving a 97% level of arrest of cells reaching the metaphase stage of mitosis.

3. Binucleate cell counts

Materials

The following materials were obtained from British Drug Houses and made up into the stock solution as under:

0.05M sucrose

0.14M sodium chloride

0.003M sodium tetraphenylboron (TPB)

0.005M sodium glutamate

0.002M sodium phosphate buffer pH 7.8

(Rappaport 1966 a and b; Rappaport and Mouze 1966)

The working solution was constituted by diluting 10 ml of the stock solution to 100 ml with distilled water (hereafter referred to as TPB solution).

Method

The rat was killed and 3 cub. mm of liver was removed immediately and minced finely with scissors in a petri-dish containing 5 ml TPB solution. Much of the blood in the liver was eliminated with the TPB solution when it was decanted from these liver fragments. The minced tissue was next added to 10 ml TPB solution in a 50 ml conical flask and stirred magnetically for five minutes. The larger fragments were allowed to sediment for two to three minutes and the supernatant containing much of the fibrous debris discarded. A further 10 ml TPB solution was then added and stirring resumed for ten minutes. At the end of this period, the contents of the flask were allowed to settle for one minute and the supernatant carefully pipetted off. This supernatant was then centrifuged

for ten minutes at 2000 rpm. The supernatant was discarded and the centrifugate resuspended in 2 ml of the above solution without TPB.

The suspensions were kept on ice until treated and appraised as follows. Three drops of Trypan blue (1% aq) were added. Following gentle agitation one drop of the cell suspension was placed in a haemocytometer. The total number of cells counted for any liver sample depended on the age of the animals used in any particular experiment. Where binucleate cells are few, as in rats up to three weeks of age, two thousand nuclei were scored. In experiments involving four-week-old animals, however, where binucleate hepatocytes are very much more numerous, only one thousand cells per liver sample were scored.

Hepatocytes were readily distinguishable from other cell types by their relatively large amounts of cytoplasm.

Investigation of the possible differential fragility of mono and binucleate hepatocytes during disaggregation

Materials and methods

An adult female Ash/Wistar rat weighing 168 grams was used for this experiment. The animal was killed by ether anaesthesia and a total of 3 cub.mm of liver removed, minced finely with scissors and washed in TPB solution. The fragments were then placed in 10 ml fresh TPB solution in a conical flask. Disaggregation was then carried out using a magnetic stirrer to mix the suspension. At 10, 30 and 60 minutes 1 ml samples were pipetted from the conical flask and replaced each time by 1 ml of fresh solution. Each sample was stained by Trypan blue as described above and three counts made with each sample of the number of mononucleate, binucleate and

isolated nuclei in the haemocytometer. The sample was kept on ice as counts were carried out.

Results

Table 2 in the Appendix sets out the individual counts observed.

Table 5.1 below illustrates that the numbers of intact cells separated increases with time, while Table 5.2 gives the percentages of binucleate cells found in each of the samples. Variance analysis failed to demonstrate a significant difference in the proportion of binucleate cells at any of the time intervals of disaggregation.

Table 5.1

Ratio of intact cells: isolated nuclei

Time	Ratio	Mean number of intact separated cells/ml
10 mins	0.36	1,970
30 mins	0.97	46,250
60 mins	1.61	86,850

Table 5.2

Proportion of binucleate cells of total
number of intact cells after varying
times of disaggregation

	10 mins % Binucleates	30 mins % Binucleates	60 mins % Binucleates
Samples 1	34.8	31.1	33.8
2	33.3	38.8	34.9
3	31.4	34.6	34.1
Means	33.2	34.8	34.1

Conclusion

Table 5.1 above clearly shows that the proportion of intact hepatocytes disaggregated increased with time as the sample was stirred. Much of the early disruption of cytoplasm was almost certainly due to the initial mincing of the tissue with scissors. This disruption accounts for the high proportion of isolated nuclei seen in the ten minute sample. For this reason the actual method used subsequently, involved discarding the first disaggregate after five minutes (as has already been described).

In spite of this variability in the proportion of intact cells separated, an analysis of the results set out in Table 5.2 above illustrated that the ratio of mononucleate to binucleate cells remained approximately constant regardless of the time of disaggregation. These results of the liver cell separation and counting technique were therefore taken to be representative of the relative proportions of each cell class actually present in the liver. Further support for this conclusion may be derived from the fact that binucleate populations of 30-35% in adult rat liver have been found by other workers utilizing cell suspension techniques (Carriere, 1962; Alfert and Geschwind, 1958; Nadal and Zajdela, 1966).

4. Microspectrophotometry

A variety of methods have been used for estimating nuclear ploidy in the liver.

It has already been mentioned in the literature review that Jacobj (1925) measured the diameter of nuclei in adult rat liver and found that they fell into approximate size classes which showed a geometric progression. This method has since been used extensively to estimate the ploidy population of hepatocyte nuclei (Clara, 1930; Beams and King, 1942; Alfert and Geschwind, 1958; Nadal and Zajdela 1966). As was indicated in the introduction however, the size of nuclei can vary somewhat due to fluctuation in extra-chromosomal protein which is unrelated to changes in DNA content. With this in mind, it is not surprising that the size of nuclei can vary quite markedly within any one ploidy class and that consequently the respective ploidy populations are by no means distinctly separated (Swift, 1950; Alfert, 1958). Moreover this technique must be carried out by scanning of histological sections, which in terms of measuring a representative population of nuclei of differing sizes is in itself fraught with difficulties (Abercrombie, 1946).

A second technique involves actual counts of metaphase chromosomes in smear preparations (Bieseke, 1944). Such a method is only really applicable to experiments which do not require estimation of the ploidy of large numbers of nuclei. This system is also open to criticism in that, only cells actually in division are evaluated and a representative population of hepatocytes may not be scored.

By far the most extensively used system involves estimation of the

actual DNA content of interphase nuclei using either ultraviolet or visible light. In the latter case the nuclei are stained specifically for DNA and the relative absorption of monochromatic light by the nuclei measured.

A number of commercial instruments have been developed for this purpose which make use of an integrated scanning mechanism of the whole nucleus. Such scanning microspectrophotometers are by far the most accurate when compared with other systems and can give errors of less than one percent.

The early instruments, as used for example, by Swift (1950), Moses (1952) and Pollister (1952), lacked many of the refinements to be found in modern instruments but were relatively easy to construct and far less expensive than commercially available scanning microspectrophotometers. Estimation of DNA of individual nuclei involves one of two basic systems. The plug method relies on measuring the absorption of monochromatic light by a small area of the nucleus. The volume of the nucleus is then estimated from the measurement of the diameter and the total DNA content thus calculated. The second method involves absorption of monochromatic light over the entire nucleus at two wave lengths; this is a more accurate technique than the plug method with which it is impossible to reduce error of measurement to less than ten percent. However, availability of apparatus necessitated the use of the plug method in the experiments of this thesis and a simple microspectrophotometer was constructed for this purpose.

Although these methods of DNA measurement can be used on histological sections it was felt that accurate scanning of hepatocyte nuclei would be virtually impossible due to the large islets of haemopoietic cells in the

liver of the neonate rat. For this reason, a technique involving measurement of DNA content of hepatocyte nuclei in smear preparations was used as validated by Karson (1951). Moreover, this method avoids errors in differential staining of nuclei in histological section related to variations in fixation of the tissue blocks (Deitch, 1966).

Instrumentation

The minimum requirement of the microspectrophotometer used in this work was to enable nuclear DNA to be measured with such accuracy that the nuclei could be placed in a particular ploidy class. It was, therefore, thought that a simple adaption of a standard bench microscope would suffice for this (Meek - personal communication). The review of simple microphotometer construction by Swift and Rasche (1953) served as an excellent guide in this respect.

The microspectrophotometer had three basic functions to fulfil.

These were:-

- i) To supply a constant and stable monochromatic illumination.
- ii) To measure the absorption of this light by an area of a nucleus which had been stained specifically for DNA.
- iii) Finally, to enable one to measure the diameter of the nucleus so that the final DNA content could be calculated.

Fig. 5.1 shows the instrument used which was based on a Vickers Patholux microscope. The output of the twelve-volt tungsten halide lamp used, was standardised by means of the constant voltage transformer A. The light passed through a field diaphragm at B and through a monochromatic filter (Balzers 546 m μ) at C.

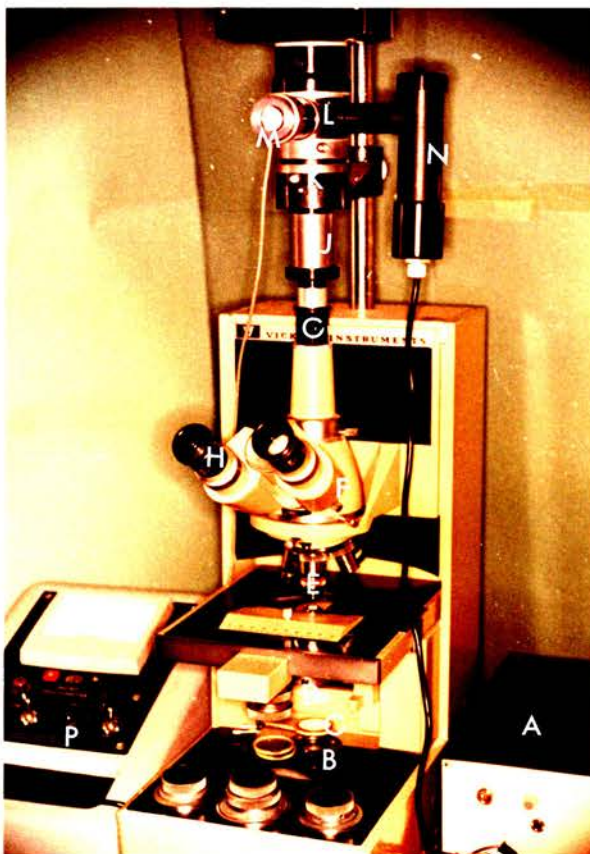


Fig. 5.1

Vickers Patholux microscope adapted for
microspectrophotometric measurement

- | | |
|-------------------------|--------------------------|
| A. Voltage stabilizer | H. Binocular eye-piece |
| B. Field diaphragm | J. Extension tube |
| C. Monochromatic filter | K. Measurement diaphragm |
| D. Achromatic condenser | L. Prism |
| E. Objectivelens x 100 | M. Telescope |
| F. Prism | N. Phototube |
| G. Ocular lens x 20 | P. Photometer |

An acromatic condenser, D, directed the light onto the area required and contained a diaphragm for altering the numerical aperture. The objective lens, E, was a x 100 acromatic and also contained an adjustable diaphragm, the light passing through here to a prism at F. The control lever of this prism directed the light either to the binocular eye piece, H, in which was inserted a graticule for measurement of nuclear diameters, or vertically through a x 20 aplanatic ocular lens at G, to the photometer head. This ocular lens, together with the extension tube, J, produced a final magnification at K of approximately x 2,500. The adapter at K, was fitted with a standard substage diaphragm. A prism at L enabled one either to view the specimen through the telescope M, or to direct the light onto the phototube N. Light falling on the phototube produced a reading on the ammeter P (EEL photometer).

Measuring technique

The microscope was adjusted to give Köhler illumination of the specimen and carefully aligned so that the centre of the field as seen at the binocular eye-pieces exactly coincided with the centre of the diaphragm aperture at K and with the centre of the telescope viewer M. As much extraneous flare was eliminated as possible from the light source and specimen by closing down the field diaphragm B to a minimum, the substage iris condenser to an aperture of 3 mm, and limiting the aperture of the diaphragm in the objective lens, E, to approximately half its potential size. The iris diaphragm, K, was stopped down to its minimum aperture so that it encompassed an area which represented a diameter of 2.5μ at the level of the specimen.

In practice the nucleus was centred in the microscope field, as viewed at the binocular eye-pieces, and its diameter measured at two right angles by means of the eye-piece graticule. The light was then directed vertically and the reading noted on the photometer. This was then repeated on an area just off centre of the nucleus and the mean nuclear absorption calculated from these two readings. Two equivalent areas were then measured immediately adjacent to the nucleus to give the mean background reading. From these observations the nuclear DNA content of the nucleus could be calculated in arbitrary DNA units.

Calculation of results

The determination of light absorption by DNA, as indicated above, involves two measurements; these are the intensity of incident or background light (I_0) and the light intensity of the incident beam reduced by passing through the specimen (I_3).

The percent transmission, ($\%T$) is then given by
$$\frac{I_3}{I_0} \times 100$$

This transmission is a reciprocal log. function of the absorption of light by the specimen.

The extinction (E) is then defined as

$$E = \log_{10} \frac{1}{T} = \log_{10} \frac{I_0}{I_3}$$

Smears of cells produce nuclei which can be considered as flat discs (Karson, 1951) and the arbitrary DNA content, M , can thus be computed as $E \times \pi r^2$. (r = radius of nucleus).

The repetitive calculation of the DNA contents of individual nuclei from the above formula proved to be extremely tedious. A short computer-programme-writing course was therefore attended, and the programme as set out overleaf written and used for the computation of results. (See p 109)

Key: M = DNA content of nucleus

A and B = respective nuclear diameters measured
at right angles

C = background reading, I_3

D = specimen reading, I_0

This programme is written in Imp (AA) language and was run on an I.B.M. 360/50 machine.

The preparation and staining of liver cells

Liver cell disaggregation was carried out as given in the binucleate cell count technique (p 98) and one drop of the final cell suspension smeared onto a scrupulously cleaned slide (Microscope slides HJ750 0.6-0.8 mm. Gallencamp Technico House, London E.C.2.). The smear was quickly dried under a stream of warm air and then immediately fixed in 3:1 ethanol-acetic acid for one hour.

DNA was stained with a Feulgen technique as described below:-

A) Preparation of Schiff's reagent

i) 5g of basic fuchsin were dissolved by pouring over it, 1 litre of boiled distilled water. The solution was then shaken thoroughly, cooled to 50°C and filtered.

ii) 100 ml NHCl and 20g of potassium metabisulphite ($K_2S_2O_5$) were then added to the filtrate. The mixture was again well shaken and then placed in the dark for twenty-four hours.

COMPUTER PROGRAM

```
1  %LIST
2  %BEGIN
3      %REALSLONG
4      %REAL A,B,C,D,E
5      %REALARRAY M(1:100)
6      %INTEGER COUNT,N
7      %INTEGER F
8      %CYCLE F=1,1,8
9      %CYCLE COUNT=1,1,100
10     READ(A):READ(B):READ(C):READ(D)
11     M(COUNT)=LOG(C/D)*0.4343*((A+B)/4)**2*E
12     %REPEAT
13     %CYCLE N=1,1,100
14     E=M(N)
15     %IF 1<=M(N)<5%THEN ->1
16     SPACES(8)
17     %IF 5<=M(N)<10%THEN ->1
18     SPACES(8)
19     %IF 10<=M(N)<15%THEN ->1
20     SPACES(8)
21     %IF 15<=M(N)<20%THEN ->1
22     SPACES(8)
23     %IF 20<=M(N)<25%THEN ->1
24     SPACES(8)
25     %IF 25<=M(N)<30%THEN ->1
26     SPACES(8)
27     %IF 30<=M(N)<35%THEN ->1
28     SPACES(8)
29     %IF 35<=M(N)<70%THEN ->1
30     SPACES(8)
31     %IF 70<=M(N)<150%THEN ->1
32     SPACES(8)
33     %IF 150<=M(N)<300%THEN ->1
34     1: PRINT(E,3,3)
35     NEWLINE
36     %REPEAT
37     NEWPAGE
38     %REPEAT
39     %ENDOFPROGRAM
```

iii) 2.5g of neutral activated charcoal was then added, shaken for one minute and the mixture filtered rapidly through coarse filter paper. The final clear filtrate was stored in the refrigerator in a tightly closed amber-coloured glass bottle.

The solution was made up as required but was never used if it was over 6 weeks of age.

B) Preparation of sulphurous acid bleaches

600 mls of distilled water was added to a flask containing 30 ml NHCl and 30 ml 10% anhydrous potassium metabisulphite.

This solution was made up freshly each time just before it was needed.

C) Staining procedure

i) The slides were hydrated and then hydrolysed in NHCl at 60°C for ten minutes. (They were rinsed in distilled water at 60°C just prior to this to bring them to the required temperature).

ii) The slides were briefly chilled in cold NHCl .

iii) They were then transferred to a light-tight box containing Schiff's reagent where they remained for forty minutes.

iv) Following this staining procedure the slides were taken through three successive changes of sulphurous acid bleaches for a total of thirty minutes.

v) The slides were then rinsed for five minutes in running tap-water following by distilled water, counter-stained in 0.1% aqueous light-green for five seconds, dehydrated through a graded series of alcohols and finally mounted in undiluted DPX (RI/1.52).

Note on the Feulgen stain

The specificity of the Feulgen reaction for DNA would not seem to be in any doubt. The reaction is negative after the complete removal of DNA either enzymically, Brachet (1946), or by the extraction of the tissue with hot trichloroacetic acid (Schneider, 1945). RNA and protein do not give the Feulgen reaction (Feulgen and Rossenbeck, 1924; Lessler, 1951) and pretreatment with ribonuclease has no effect on Feulgen intensity (Barka and Dallner, 1959).

The Feulgen reaction has been shown to depend upon prior acid hydrolysis of DNA which preferentially removes purines, unmasking the aldehydic function of the de-oxynucleoside sugars to which they are bound (Di Stephano, 1952; Feulgen and Rosenbeck, 1924; Overend and Stacey, 1949). The aldehydes thus formed react with the decolorised Schiff reagent (Schiff, 1866), which is converted into its coloured form and bound in situ to the DNA without diffusion (Lessler, 1951; Overend and Stacey, 1949).

Tests carried out on the microspectrophotometer

The ability of the instrument described above to separate the hepatocyte nuclear population into respective ploidy classes was tested for in adult mouse liver (C3H strain - 30g body weight). The adult mouse liver has been shown to exhibit extreme polyploidy in the liver (Epstein, 1967). After liver cell disaggregation smears were prepared and stained as described above. The incidence of nuclei in a two-hundred nuclear sample within the various ploidy classes is shown in Fig. 5.2. It may readily be seen that the sample of nuclei consists of three

populations corresponding to diploid, tetraploid and octoploid classes. A single 16N nucleus was also observed.

The actual number of nuclei within any one class was estimated by taking the intermediate point between the means of any two populations as indicated by the 3N, 6N and 12N markers in Fig. 5.2. The nuclei falling between any two adjacent intermediate points were then ascribed to the appropriate ploidy class.

From the above measurements, the proportions of nuclei in each of the respective ploidy classes were found to be:-

2N	28.5%
4N	56.0%
8N	15.0%
16N	0.5%

Karson (1951), by measuring extinctions of one nucleus with absorption plugs of varying diameters was able to report that nuclei of haemopoietic cells in smear preparations could be considered as flat discs.

This type of investigation was carried out on nuclei from the liver smear preparations. It was concluded that hepatocyte nuclei in smears also behave optically as flat discs. The relationship between the diaphragm aperture at K (see Fig. 5.1) and the phototube response, as evaluated on a uniformly lighted background area, is seen in Fig. 5.3. Over the first three apertures the response is linear. Fig. 5.3 also gives the extinction of the same nucleus as found with the appropriate plugs A, B and C (see Fig. 5.4 below) at each of these apertures.

Fig. 5.2 Histogram showing distribution of mouse hepatocyte nuclei according to their DNA count

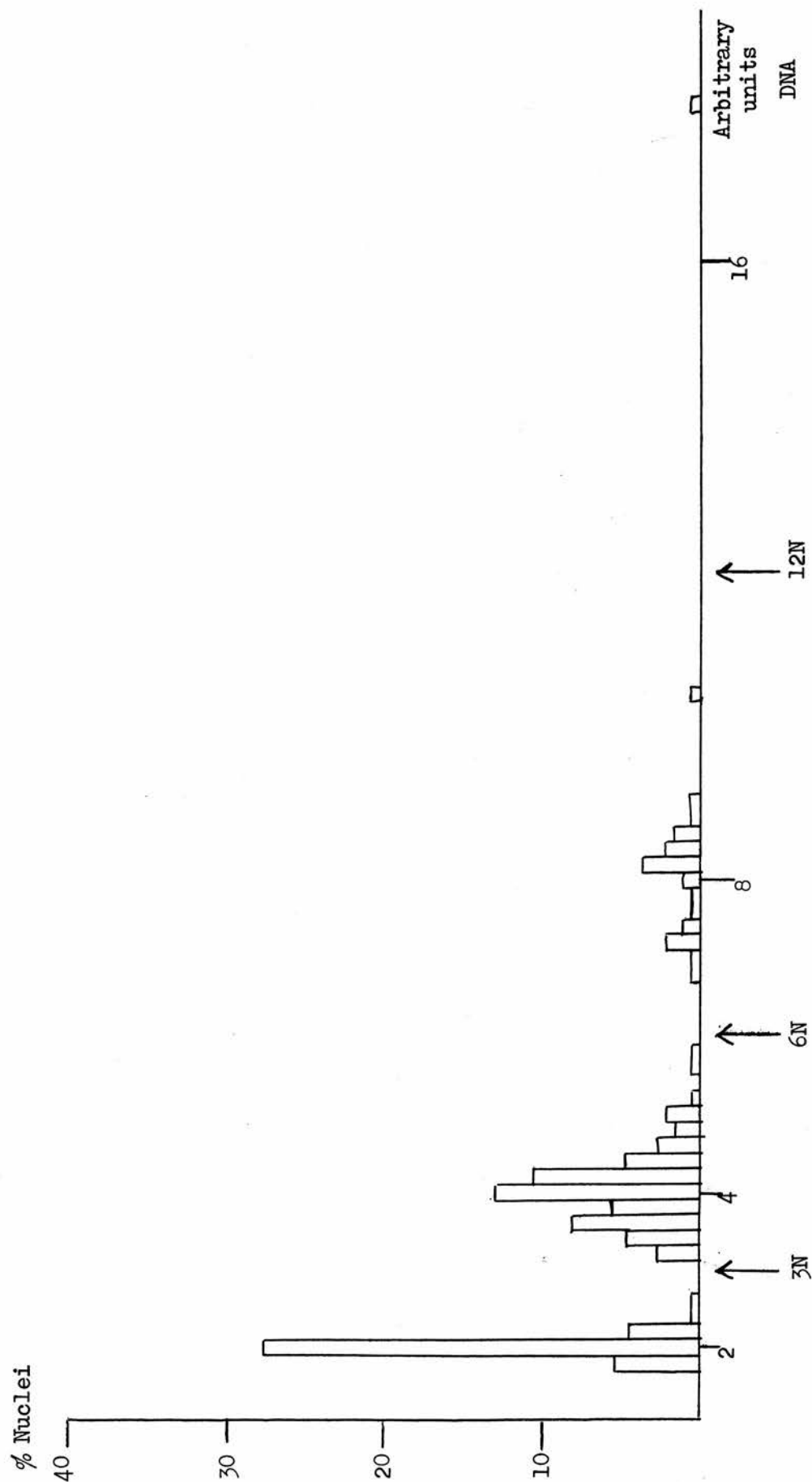


Fig. 5.3 Graphical representation of the relationship between photometer reading and size of measuring aperture

(extinction values obtained at appropriate diaphragm apertures are given in brackets).

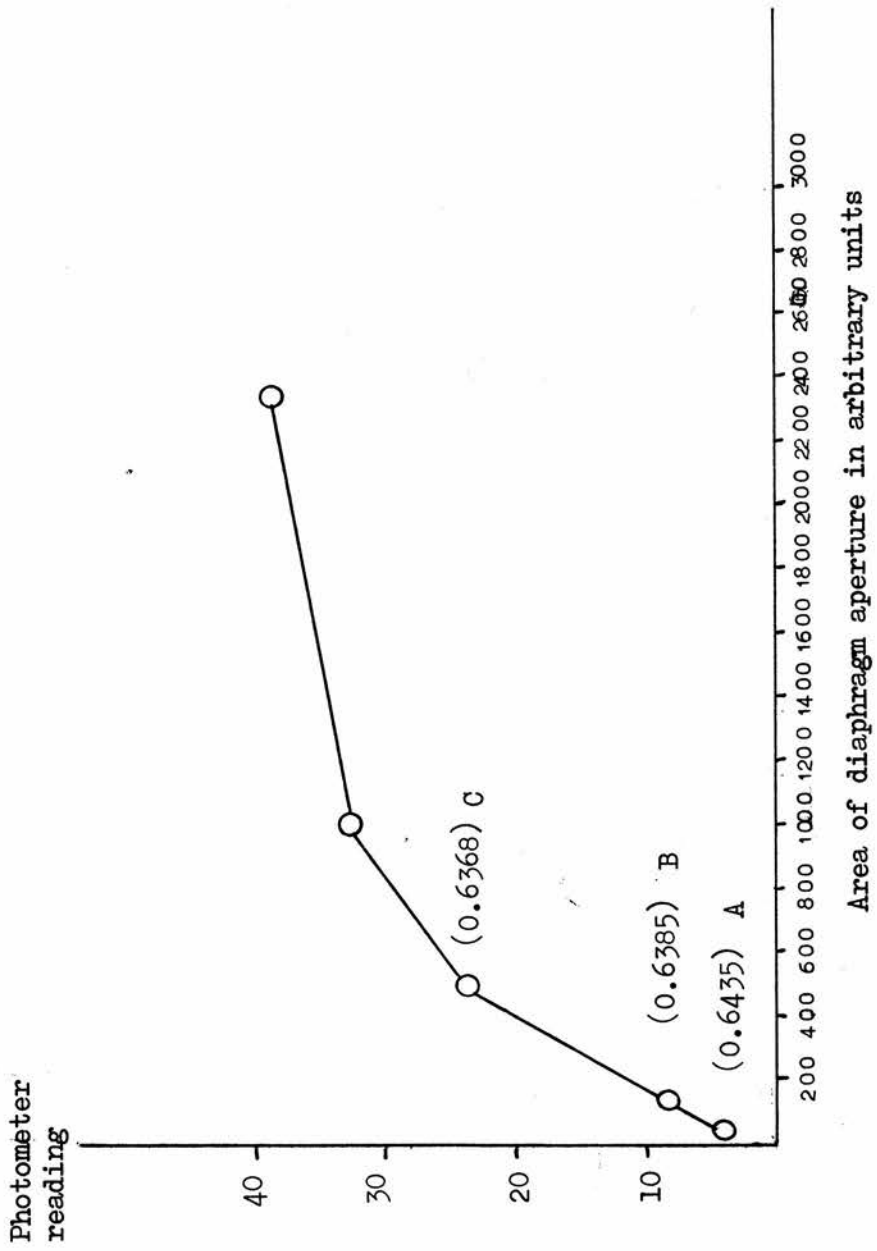
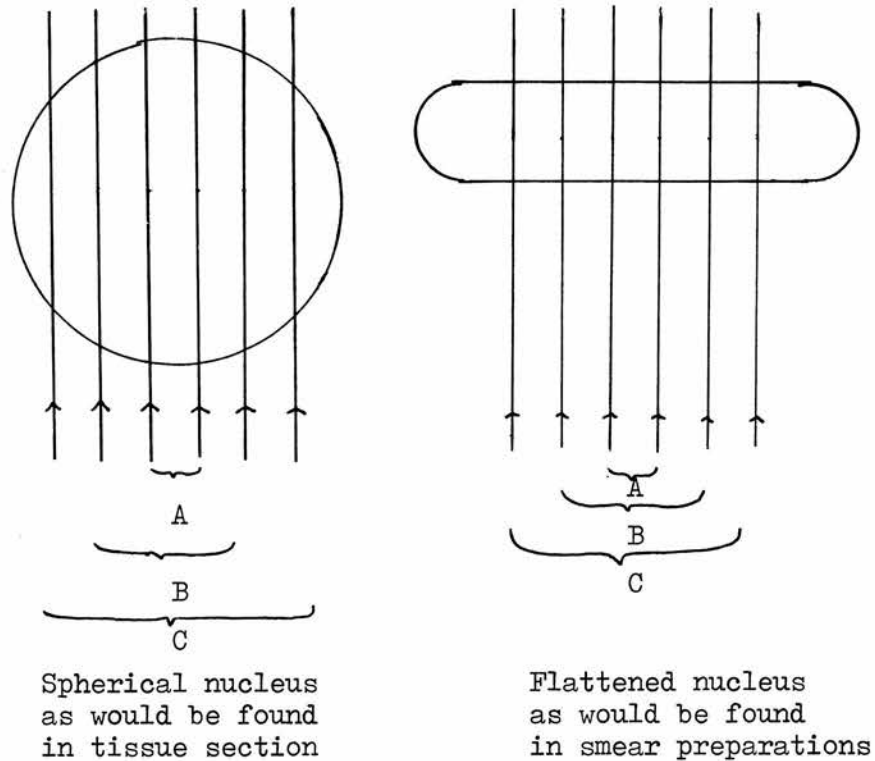


Fig. 5.4 Appearances of cross-sections of nuclei in tissue-section and smear preparations



On examination of the above diagrams it can be seen that if the nucleus is in the form of a sphere, an extinction measured through plug A, is greater than that measured through plug B which in turn is greater than that measured through plug C. A nucleus represented by a flat disc however, gives similar extinctions at all three apertures. The area of the plug measured at C (Fig. 5.4) was approximately sixteen times as great as that of A but the extinction of the two apertures were nevertheless, very similar. (See Fig. 5.3)

Interpretation of results

The significance of the microspectrophotometric results obtained above and in subsequent experiments depended on the degree of error inherent in the measurement of nuclear diameters and extinctions. The variables involved in the estimation of both of these parameters were

discussed in detail by Swift and Rasche (1953) and include:-

i) Error in measurement of nuclear diameters

Where determinations of nuclear diameter are used to compute nuclear area, any error is squared. Such error becomes greater with decreasing sizes of nuclei.

The mean diameter for the diploid nuclei in Fig. 5.2 was approximately 6 arbitrary units to the nearest 0.25 of a division as measured with the ocular graticule.

Thus the maximum error in this measurement would be approximately

$$\frac{0.25}{6} \times 100 = \pm 4\%$$

ii) Error in absorption measurements

(a) Variables from the specimen

I. Light scatter was minimised as far as possible by using scrupulously clean, thin slides and mounting the smears in a minimum of undiluted DPX so that the refractive index of the latter would be lowered as little as possible. Such scatter results in light being deflected out of the measured path with a consequent lowering of extinction values.

2. Error due to non-random dye distribution in the nucleus was unavoidable although it was eliminated to some extent by taking the mean of two readings through the nucleus.

(b) Variables from the instrument

I. The use of a monochromatic filter rather than a monochromator brings in heterochromatic light error which tends to lower extinction values.

2. Flare in the optical system was tested for by measuring the extinction values of completely opaque pyknotic nuclei in sections stained haematoxylin and eosin. Values between 1.9 and 2.1 were obtained at the lowest practicable condenser diaphragm aperture. Thus in this respect the instrument fell somewhat short of requirements as extinction values should exceed 2.5 if a flare error of less than 0.3% is to be acquired. Flare produces less error at low extinctions e.g. below 0.5. Extinctions of nuclei in this work always fell between 0.2 and 0.7 which would have helped to minimise error due to flare.

With regard to the accuracy of the above technique it is worth while finally, to quote Mendelsohn (1966) who stated that with his experience in microspectrophotometry the plug method resulted in an error between ten and forty percent, although the exact measurement of such error in any system was not possible.

Thus, although the mouse smear experiments reported above demonstrated that the instrument was capable of separating nuclear hepatocyte populations into their relative ploidy classes, it would not seem valid to attribute significance to nuclei with DNA contents which placed them mid-way between any two classes in the appropriate experiments of this thesis.

iii) Errors arising from scanning the smear preparations of liver cells

I. One source of error may arise from a non-random selection of hepatocyte nuclei for subsequent microspectrophotometric measurement. The procedure adopted was to select only those nuclei which fell within or touched the 20 unit division on the eye piece graticule as the section was

scanned in a vertical direction. This nucleus was then centred and measured for DNA content and the scanning continued from this point until the 20 unit length of the graticule encountered the next nucleus. This in turn was centred for measurement and scanning again continued from this point. This procedure eliminated observer bias. However, it must be admitted that there was more chance of encountering the large nuclei in the preparation since they obviously occupied a greater area.

2. A second source of error could arise from the **selective** destruction of any ploidy population of liver cells by the disaggregation technique. A control experiment investigating this point is described in Chapter 8 where it will be seen that such selective destruction did not occur.

3. In the scanning of smear preparations from livers of rats up to 14 days of age there arose the problem of differentiating between haemopoietic cell nuclei and parenchymal cell nuclei. The very faint counter-staining of the cytoplasm by aqueous-light green facilitated this differentiation. Only those nuclei contained in sufficient cytoplasm to enable one to identify them as hepatocyte nuclei were measured for DNA content.

5) Autoradiography

Materials

1. Ilford nuclear research emulsion K.2. (Ilford, Essex)
2. Amidol developer

Sodium sulphite ($8H_2O$)	2.20 g
Sodium hydrogen sulphite (SG. 1.34)	0.46 ml
Amidol	1.00 g
Distilled water	620.00 ml

Filtered and used immediately.

3. Safe-light. Ilford S902 orange-brown
4. Tritiated thymidine. Thymidine (methyl H^3) 5Ci/m mole
(Radio chemical Centre, Amersham, Bucks.)

Method

The animals were killed and a 4 mm thick slice of liver from each individual was removed and fixed in Carnoy's fluid for one hour and then transferred to 70% ethanol to await processing. Processing was carried out by hand using fresh materials, the tissue being dehydrated through a series of alcohols, cleared in chloroform and embedded in paraffin wax. Sections were cut at 4 μ and floated onto chromic-acid-cleaned, gelatinized slides. The sections were dried, dewaxed in clean xylene and hydrated through a series of clean alcohols. The slides were then coated with emulsion. (The use of clean glassware and freshly prepared chemicals throughout was found to be essential in keeping background on the processed autoradiograms to a minimum).

To prepare the emulsion, 0.5 ml glycerine was mixed with 19.5 ml distilled water to form a diluent which was heated to 43°C in a water bath. A quantity of gel, sufficient to give 20 ml liquid emulsion was placed in a dipping jar (a 100 ml measuring cylinder cut at the 5.0 ml mark). The emulsion was allowed to liquify in a water-bath at 43°C in total darkness. The heated diluent was then added to the emulsion and the mixture gently stirred. The diluted emulsion was allowed to mix for five minutes in the water bath before any slides were coated.

The slides were coated by removing them from the water, draining them vertically onto filter paper and placing them in a dipping jar. The slides were moved up and down withdrawing $\frac{1}{2}$ - $\frac{3}{4}$ of the slide length from the emulsion, three times at approximately 2-3 second intervals and then removed slowly and smoothly from the emulsion. The slides were drained vertically for five seconds and the excess emulsion wiped off the back of the slide with tissue paper. The drained slide was then placed horizontally on a cooled inverted aluminium ice-cube tray. The slides were cooled for at least two minutes before being placed vertically against a wooden L-shaped rack to air dry. The slides were air-dried in total darkness for 20-30 mins. and then placed in a sealed air tight, light-tight box. A positive control and a fogged non-radio-active slide were included with the experimental slides. Using this technique it was possible to dip fifty slides in 1 hour without reheating the emulsion. The slides were exposed, without desiccants for three weeks at 4°C.

Following exposure, the slides were developed for seven to eight minutes, rinsed for thirty seconds in distilled water, fixed in thirty percent sodium thiosulphate for ten minutes and after rinsing, finally stained with methyl green pyronin as given on p 150.

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[Note: In Section III photographic plates may be found at the end of each chapter and are referred to in the text as Figures. Graphs, histograms etc. are incorporated into the text of each chapter and are referred to as Text-figures.]

CHAPTER 6

PRELIMINARY STUDIES WITH DIMETHYLNITROSAMINE
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A. Pilot study

Experiment 6.1

The following brief account describes the preliminary experiment from which stemmed the remainder of the work of this thesis: it is not intended to elaborate to any great extent on the results or discussion of this experiment as the findings are dealt with in detail elsewhere (see Section B of this chapter). The two objectives of the experiment were to evaluate both the DNA content of hepatocyte nuclei and the histological changes following a single dose of dimethylnitrosamine given to the neonate rat. The dose level of DMN chosen for this experiment was based on the published figures for the LD₅₀ of the compound in the adult rat (Heath and Magee, 1962). It was hoped that this level of administration would result in a large percentage of survivors in the treated group.

Method

i) Ploidy evaluation

A litter of 13 Carworth/Wistar rats was divided into two groups at two days of age. One group, consisting of nine rats was dosed intraperitoneally with 20 mg DMN/kg body weight and the remaining four rats, which formed the control group, received 20 µl physiological saline by intraperitoneal injection. The animals were sacrificed 14 days following injection, the livers were disaggregated, smears being prepared and stained by Feulgen's method (p 108). Fifty hepatocyte nuclei from each animal were measured with respect to their DNA content.

ii) Histological evaluation

A litter of 14 Carworth/Wistar rats was divided into two groups at two days of age. One group, consisting of ten rats (5 males and 5 females), was dosed with 20 mg DMN/kg body weight and the remaining four rats, forming the control group, received 20 μ l physiological saline by intraperitoneal injection. The animals were sacrificed in groups of one control rat and two DMN dosed animals at one day, two days, three days, six days and fourteen days following the administration of DMN. After macroscopic examination the liver, kidneys, spleen, stomach, small and large intestine, heart and lungs were removed from each animal and fixed immediately in ten percent neutral buffered formalin. After adequate fixation, blocks of these tissues were routinely embedded in paraffin wax, sections cut at 4 μ and stained with Ehrlich's alum haematoxylin and eosin.

Results

i) Ploidy evaluation

One male and one female from the group dosed with dimethylnitrosamine died on the first and the fourth day of the experiment respectively, leaving the remainder to be sacrificed on the fourteenth day.

Table 6.1 gives the hepatocyte nuclear ploidy classes of individual rats, together with the means of each group.

Table 6.1

Distribution of the percentage of the hepatocyte nuclei in each ploidy class (Carworth/Wistar rats dosed at 2 days old and killed when 14 days old)

Treatment	Rat No.	% 2N	% 4N	% 8N
Control group (20 µl saline ip)	1 M	92	8	0
	2 M	92	8	0
	3 F	100	0	0
	4 F	98	2	0
	Mean	95.5	4.5	0
DMN treated group (20 mg DMN/kg body weight ip)	1 M	54	46	0
	2 M	54	42	4
	3 M	66	34	0
	4 F	60	32	8
	5 F	66	32	2
	6 F	62	38	0
	7 F	56	40	4
	Mean	59.7	37.7	2.6

2N = diploid

M = Male

4N = tetraploid

F = Female

8N = octoploid

The actual distribution of the hepatocyte nuclei with respect to their DNA content is shown in the histograms of Text-Fig. 6.1.

ii) Histological evaluation

Only livers from those rats treated with DMN appeared abnormal. The changes seen will be described chronologically.

The livers of the control animals were as described in the detailed appraisal of Experiment 6.3 (p 136).

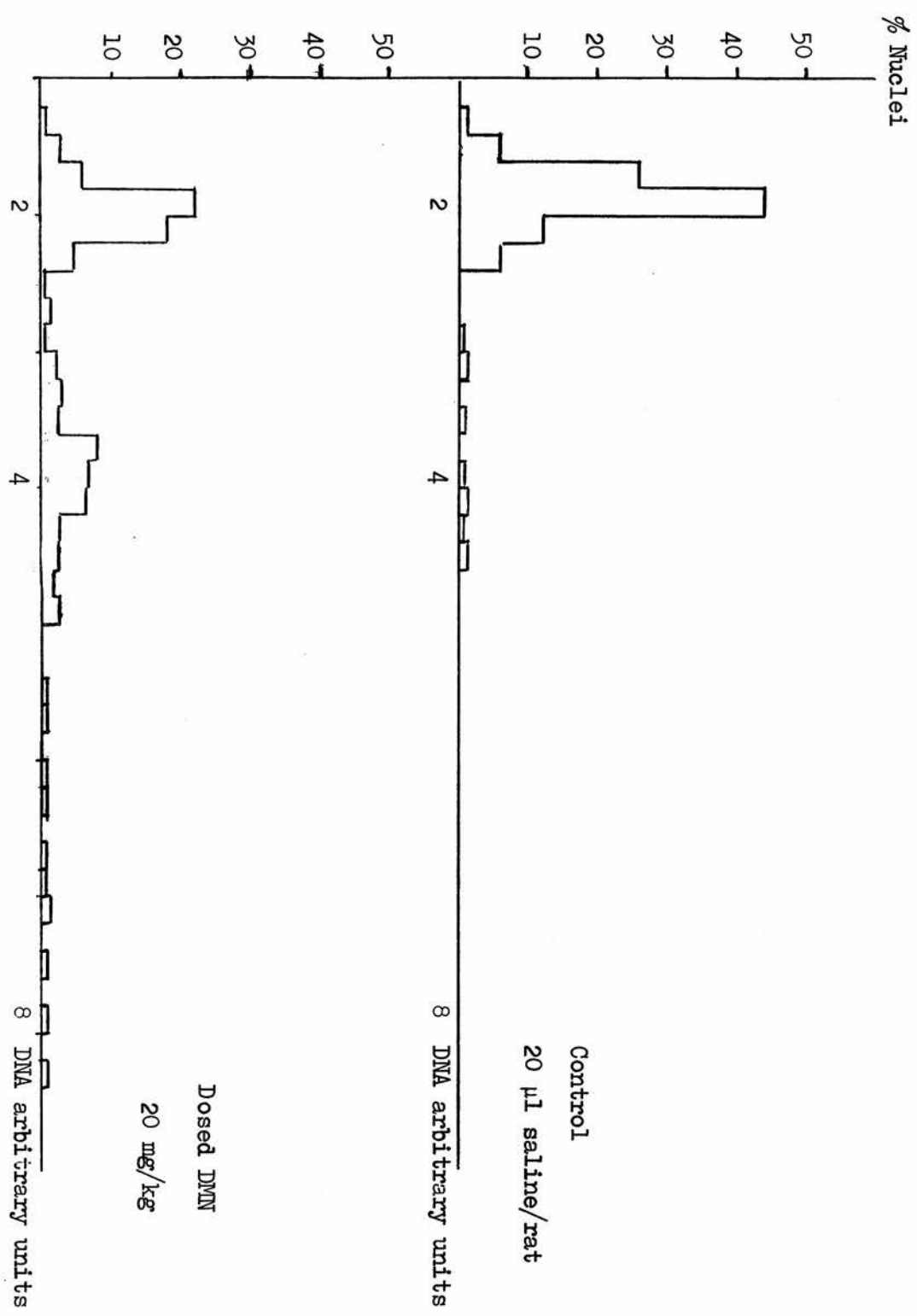
Day 1

Gross vacuolation of centrilobular hepatocytes was apparent and these regions were pale when compared to areas immediately surrounding the portal tracts. Although, as will be seen in the description of the control livers given in Experiment 6.3., it is difficult to ascribe a lobular pattern to the neonate liver during the first week of life, these regions of vacuolated hepatocytes occupied what would be termed the central and midzonal areas surrounding the primitive central veins. The liver cell nuclei were more variable in size than those of the control liver and had a more irregular outline. This variability in nuclear size appeared to be uniformly distributed throughout the liver and remained so for each group throughout the course of the experiment.

The haemopoietic tissue, which was abundant at this time in the control livers, showed gross depletion in the livers of the treated group. Occasionally, haemopoietic cells showed necrotic changes in that pyknotic and karyorrhexic nuclei could be seen.

Text-Fig. 6.1

Distribution of liver parenchymal nuclei according to DNA content in 14-day old control and DMN treated Garworth/Wistar rats dosed at 2 days of age.



Day 2

The areas of palor containing vacuolated hepatocytes still persisted and the nuclei were yet more variable in size.

The haemopoietic tissue was by this time almost completely absent with the occasional single isolated cell persisting in the sinusoids. All elements of the haemopoietic tissue, erythrocytic, myelocytic and megakaryocytic appeared to be involved in this depletion.

Day 3

The liver still showed slight vacuolation of hepatocytes centrilobularly. The nuclei of the liver cells remained variable in size and the haemopoietic tissue was still almost entirely absent but the presence of one or two islets of approximately five to ten cells, many in mitosis, indicated the onset of regeneration at this time.

Day 6

The centrilobular areas of the livers of the dosed animals by now contained hepatocytes with uniform staining of the cytoplasm and the pronounced vacuolation seen earlier was absent.

Regeneration of the haemopoietic tissue had been extensive between the third and sixth day following the administration of dimethylnitrosamine since large islets were now seen scattered throughout the liver parenchyma.

In the liver section from one of the rats killed on the sixth day, very large groups of haemopoietic cells, with round large vesicular nuclei were observed. The cytoplasm was scanty, fairly homogenous and slightly basophilic. This type of cell was not seen

in the liver of any other neonate rat throughout the remainder of the work of this thesis. Many such cells were found in the spleen, however, and those in the liver were thought to be of the lymphocytic series (Fig. 6.1).

Day 14

The livers of the dosed animals had a similar appearance to those of the control animals apart from the variability in nuclear size of the hepatocytes.

Conclusions

The ploidy evaluation showed a marked shift of DNA content of hepatocyte nuclei in the dosed animals as compared with controls. The almost homogenous diploid distribution of the control hepatocyte nuclei provided an excellent base-line for comparison.

The histological study afforded evidence that, at a dose level of 20 mg/kg body weight in Carworth-Wistar rats, DMN did not cause necrosis of hepatocytes, although pronounced cytoplasmic changes were observed. In addition there was gross depletion of the haemopoietic tissue in the liver.

The large proportion of Carworth-Wistar neonate rats which survived the DMN treatment indicated that 20 mg/kg would be a suitable dose level to use for future experiments. However, at this point in the work, for animal housing reasons, the laboratory changed over to Ash-Wistar rats as breeding stock. Experiments were therefore set up in these rats to determine their susceptibility to DMN. Unfortunately 20 mg/kg DMN caused 100 percent mortality in all the litters used. It thus became necessary to evaluate the dose response of DMN in Ash-Wistar rats.

B. Preliminary studies with DMN in Ash-Wistar rats

As outlined in the experiment above, in which Carworth-Wistar rats were used, it became necessary to evaluate the dose response of the Ash-Wistar strain to DMN.

The first experiment therefore investigates the LD₅₀ of DMN in the latter strain of rats at two days of age.

As a result of this experiment 10 mg/kg DMN was chosen as a suitable dose level and the histological changes seen at this level will be described.

The third experiment was set up in an attempt to investigate the nature of the necrotoxic effect of DMN on the haemopoietic tissue in the neonate liver.

Experiment 6.2: To evaluate the LD₅₀ of DMN in two day old
Ash-Wistar rats

Method

Two-day old Ash-Wistar rats were allocated to one of four groups and dosed with 5, 10, 20 or 40 mg DMN/kg body weight by intraperitoneal injection. This procedure was repeated as litters became available so that five males and five females, and approximately equal numbers of rats from each litter, were represented at each dose level. The time of death of any individuals was noted (some animals were cannibalised, see table below, and it was assumed that death had occurred prior to this event). The median-lethal dose was then calculated according to the method of Weil (1952).

Results

Table 6.2 sets out the numbers of deaths in any day following administration of dimethylnitrosamine.

Calculations:

$$\text{Log } m = \log D_a + d \cdot (f + 1) \text{ for } K = 3 \text{ [K = No. of dosage levels -1]}$$

where $m = LD_{50}$

D_a = log of lowest dose used

n = number of animals dosed in each group

d = log of constant ratio between dosage levels

f - obtained from tables of Weil

95% confidence limits $\pm 2:d.df$ where df obtained from tables.

i) Males 0, 0, 4, 5

$$\log m = \log 5 + 0.3010 (0.7 + 1)$$

$$m = 16.2$$

with 95% confidence limits $\pm 2(0.3010) (0.2) = \pm 0.12$

$$LD_{50} \text{ DMN in male neonate rats} = 16.2 \pm 0.12 \text{ mg/kg}$$

ii) Females 0, 0, 5, 5

$$\log m = \log 5 + 0.3010 (0.5 + 1)$$

$$LD_{50} \text{ DMN in female neonate rats} = 14.1 \text{ mg/kg} \pm 0 \text{ mg/kg.}$$

Table 6.2
Survival of rats following administration of DMN at 2 days of age

Sex	Dosage level (mg DMN/kg body weight)	No. of deaths on any day following administration DMN						Survival at Day 7
		Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	
Male	5							5
	10							5
	20	2	1	1				1
	40	3 ^c	2 ^c					0
Female	5							5
	10							5
	20	5						0
	40	5 ^c						0

c = one rat cannibalised

Discussion

On the basis of both the calculations above, and the difference in duration of survival as shown in Table 6.2., it would seem that the female neonate rat is more susceptible to the lethal effects of DMN than the male at this age. However, it will be recalled that the accuracy of the dosing technique (see materials and methods p 90) would place the validity of this difference in some doubt.

In comparison to the Carworth strain of Wistar rats, however, which were able to withstand 20 mg/kg with almost one hundred percent survival, it is apparent that the Ash strain were considerably more susceptible to the lethal effects of DMN.

LD₅₀ values for dimethylnitrosamine in the neonate rat have not been found in the literature although levels of 26-40 mg/kg have been given as the LD₅₀ in adult albino rats (Heath and Magee, 1962). However, Terracini and Magee (1964) were able to give 125 μ l DMN to both newborn rats (less than 24 hours of age) and seven day old rats with virtually one hundred percent survival. On my own experience concerning the association of age and weight gained with Ash-Wistar rats, the administration of DMN in the above work of Terracini and Magee would represent dose levels of approximately 25 mg/kg in the newborn rat, and 7.5 mg/kg at a week of age. More recent work (Terracini, 1969) has shown that rats of the Porton strain of Wistar rats were also able to withstand 125 μ l DMN at less than twelve hours of age. On this basis, therefore, it would appear that the Carworth strain used in the pilot experiment of this thesis could have been showing the same order of susceptibility as those strains used in the

above reported studies. In contrast, experiment 6.2 above has shown that the Ash-Wistar strain is considerably more susceptible to the lethal effects of DMN. It will be seen in the next experiment that the lethal effect of DMN is almost certainly due to the gross liver damage induced by this hepatotoxic chemical.

Experiment 6.3: Histopathological changes seen in the liver of the two-day old rat after DMN administration

Method

Two-day old Ash-Wistar rats were dosed at 10 a.m. with either 10 mg DMN/kg body weight or 20 μ l physiological saline by intraperitoneal injection and killed at daily intervals until the sixth day, and then on the ninth, fourteenth and twenty-first day following injection. The death of the animals was arranged so that at least one female and one male from the DMN dosed and the control group were killed at each time interval. In addition, two male and two female rats were dosed with 20 mg DMN/kg body weight at two days of age and the survivors killed 24 hours later. Finally, in order to compare the necrotoxic effect of the compound in the neonate and the adult, one adult female rat was given 30 mg DMN/kg body weight by intraperitoneal injection and killed with ether 24 hours later.

Following the killing and macroscopic examination of the rats, the liver, kidneys, spleen, lungs, heart, stomach and intestine were removed and fixed in 10% neutral buffered formalin. Blocks of the above tissues were routinely processed and embedded in paraffin. Sections were cut at 4 μ and stained routinely with Ehrlich's alum haematoxylin and eosin. Selected sections were stained for reticulin by James'

method, for collagen by the method of Van Gieson and for haemosiderin using Perl's Prussian Blue technique.

Small portions of liver and kidney were also processed for lipid as under (Drury and Wallington 1967).

- 1) Small 2 mm thick slices of liver were fixed in 10% neutral buffered formalin for up to 24 hours.
- 2) These were then post-fixed in freshly prepared Flemming's fluid for 48 hours.

1% chromic acid, 5 ml	}	Flemming's Fluid
2% aqueous osmium tetroxide, 20 ml		
glacial acetic acid, 5 ml		

- 3) The tissue was washed overnight to remove excess fixative, processed to paraffin blocks, sections cut at 4-5 μ and mounted in DPX.

Results

(i) Survival

Of the animals treated at a dose level of 10 mg DMN/kg, one animal was cannibalised 15 days after injection.

Of the animals treated with 20 mg/kg, one female and one male were found dead 24 hours after treatment. The remaining two animals in this group were comatose at this time.

The adult rat dosed at a level of 30 mg/kg was killed 24 hours following injection.

(ii) Macroscopic findings

a) Control rats

The livers of rats two to six days of age, appeared a somewhat

darker brown than they did at later ages.

b) Neonate rats administered 10 mg DMN/kg body weight

During the first three days of the experiment the livers from the treated animals appeared appreciably paler than the appropriate control animals and had a slightly mottled appearance. In addition, the liver of one of the treated rats, when examined on the sixth day, showed a dark red lesion, approximately 2 mm across. This was present on the border of the medial lobe and on cutting was found to extend approximately 2 mm into the organ. All the animals in both control and treated groups had been suckled by the dams as was evidenced by full stomachs seen at post mortem examination.

c) Neonate rats administered 20 mg DMN/kg body weight

The external appearance of the animals was one of extreme pallor of the skin in both the comatose and the dead animals. All four rats showed traces of serosanguinous fluid in the abdominal cavity. The liver appeared dark red in colour with rounded borders and had a friable consistency. The cut surface of the liver was engorged with blood. No abnormality was noted in any other organ except the large intestines. These contained black, viscous, faecal material. The stomachs of all these animals were not as distended with milk as those of the control and 10 mg/kg treated animals.

(iii) Histological findings

1) Control livers

At three days of age the liver presented, microscopically, a flat sheet of hepatocytes in which were seen large blood vessels associated with bile ducts, isolated large venous sinusoids and relatively

infrequent small intrahepatic veins. Extramedullary haemopoiesis was abundant at this time. Over the following 21 days the lobular pattern of the adult liver was assumed and the haemopoietic activity decreased considerably. It is proposed to deal separately with the individual components of the liver over this period.

(a) Hepatocytes

In the three to six-day old rats studied the cytoplasm of the parenchymal cells was markedly vacuolated and cell outlines by no means distinct. The vacuolated cells noted in the formalin fixed blocks could be demonstrated, in the blocks post-fixed with Flemming's Fluid, to contain a component which was stained with the osmium tetroxide. From the ninth daily interval the vacuolation of hepatocytes was less marked. The nuclear size, as evaluated subjectively, showed little variation up to the 23rd day of age.

(b) Hepatic vessels

The large blood vessels and associated bile ducts observed in the three-day old animals were taken as representing early portal tracts, and the small vessels isolated in the liver parenchyma as representing primitive central veins. Both these components became more frequent with age, so that by 11 to 16 days of age a lobular pattern was established, with hepatocytes assuming a cord-like arrangement radiating from the central vein. The large venous sinusoids seen at three days of age became relatively less frequent, although they were still present to some degree at 23 days of age, when they were taken to represent interlobular hepatic veins.

(c) Reticular tissue

At three days of age, the reticulin framework of the liver is not well developed apart from the reticulin fibres surrounding major blood vessels and the bile-ducts. By eight days of age however, the reticular framework throughout the liver parenchyma has developed to a much greater degree and this component was seen to increase slightly over the following two weeks.

(d) Connective tissue

The connective tissue in the liver as evaluated from the Van Gieson stained sections was also seen to increase in amount over the three week period studied. From three days of age, when it was negligible in extent, this liver component was seen to gradually become more pronounced, but was confined entirely to the portal tracts of the liver over this period.

(e) Haemopoietic tissue

The activity of this component of the liver was pronounced at three days of age. There appeared to be three configurations of the haemopoietic tissue with respect to the general architecture of the liver.

i) Islets comprising 5-50 haemopoietic cells were scattered throughout the organ. These cell aggregates were composed, mainly, of normoblasts, although small groups of myelocytes could occasionally be identified.

ii) The other major site of extramedullary haemopoiesis in the liver was sited around portal tracts and here the impression was formed

that myelocytic activity was more abundant.

iii) Throughout the sinusoids in the remaining areas of liver parenchyma, isolated cells of either series were recognisable. Megakaryocytes were also occasionally encountered and these too were usually in isolation from the main islets of erythropoiesis or myelopoiesis.

2) Histological appearance of livers from animals
administered 20 mg DMN/kg body weight

The only areas of apparently normal liver were those immediately surrounding portal tracts (Fig. 6.2). The cells in this area showed relatively basophilic cytoplasm and a variability in nuclear size. Surrounding this area was a zone of hepatocytes approximately two to three cell layers in extent with vacuolated and very pale staining cytoplasm containing small yellow pigment granules. This pigment was shown to be haemosiderin by means of Perl's Prussian Blue staining technique. In the remainder of the liver there appeared to be a considerable loss of hepatocytes with engorgement of sinusoidal spaces. The parenchymal cells in these areas showed very faintly staining cytoplasm and nuclei and some cells were necrotic as evidenced by nuclear pyknosis, karyolysis and karyorrhexis. Mitoses were not a frequent finding in the apparently viable cells surrounding the portal tracts. The haemopoietic tissue was considerably depleted and osmium tetroxide staining showed that a moderate degree of fatty change was present in the periportal cells.

Sections stained by James' method for reticulin showed a well preserved pattern of reticulin fibres remaining even in the centrilobular areas in which the liver cell cords appeared to be disrupted

in haematoxylin and eosin stained sections.

No abnormalities were found on histological examination of the remaining tissues, apart from in the spleen which showed slight engorgement of the red pulp.

The changes in the large intestine seen macroscopically were observed histologically, to be due to haemorrhage from the mucosa in both small and large intestines.

3) Histological appearance of livers from animals
dosed with 10 mg DMN/kg body weight

a) Necrosis

DMN treatment had induced severe degenerative changes in the liver by 24 hours. The distribution of these lesions was not even throughout the liver, however, and the impression was gained that they increased in extent towards the hilus of the organ. The areas affected were associated with 'central' veins but occasionally large confluent areas of early coagulative necrosis were observed (Fig. 6.3) with a sharp demarcation between degenerative and unaffected areas. Within the affected zones the cell-cord arrangement was less distinct and the sinusoids were slightly engorged. The hepatic cell size was much the same as normal but the cytoplasm was a less intensely staining faint purple as contrasted with the deep pink of hepatocytes in unaffected areas. The nuclei of degenerating hepatocytes were in most instances slightly shrunken and pyknotic, although nucleoli were still visible. There was little incidence of karyorrhexis at this time.

By the second day, these areas of degenerative change had developed into overt coagulative necrosis. The well demarcated borders of the

lesions had been maintained but pyknosis, karyorrhexis and karyolysis could now be seen throughout the necrotic areas. There was also a moderate infiltration of polymorphonuclear leukocytes by this time. The reticulin framework was still well preserved in these areas.

No evidence of necrosis of the parenchyma could be found from the third day of the experiment onwards apart from a large area in the liver section of one animal killed on the sixth day of the experiment. This lesion, already mentioned in the macroscopic description, was seen histologically to be a large area of necrosis which was separated from the remainder of the lobe by an extensive zone of fibroplasia and bile duct proliferation. The reticulin framework had been largely lost in this area and it was considerably engorged with intact red blood corpuscles.

In spite of the necrosis observed over the first two days of the experiment, mitoses of hepatocytes were by no means frequent. Apart from the lesion seen in the liver of a rat after five days, no increase in reticulin or fibrous tissue was observed in any area of the liver over the three week period studied.

b) Nuclear size

Nuclear size, in the areas of liver not undergoing necrosis, was noticeably more variable by the second day following injection and further increases occurred so that by the fourteenth day following injection relatively large hepatocyte nuclei were observable (Fig. 6.4).

c) Osmium stain

Increase of osmophilic droplets in the cytoplasm of the midzonal and periportal hepatocytes was observed from the second to the fifth day in the livers of the DMN dosed animals.

d) Haemopoietic tissue

The haemopoietic tissue was depleted over the first two days studied but showed signs of recovery by the third day and had returned to normal by the sixth daily interval studied. Its decrease then paralleled the haemopoietic tissues of control livers.

No other abnormalities were found in any other tissue taken for histology.

4) Adult rat

Histologically, the liver showed gross regular centrilobular necrosis with loss of hepatocytes, pooling of blood in this area and slight neutrophil polymorphonuclear infiltration. Surrounding this area was a small zone of hepatocytes with grossly vacuolated cytoplasm. These vacuoles stained positively with osmium tetroxide. The extent of the lesions was clearly demarcated, as in the neonate rat, and the midzonal and periportal cells seemed unaffected by the treatment. The appearance of the adult liver following this necrotoxic dose of DMN may be seen in Fig. 6.5. No abnormalities were noted in the remaining tissues taken from this animal for histological examination.

Discussion

A number of facts clearly emerge from this experiment concerning the nature of the acute lesions induced by DMN in the liver.

In the Ash-Wistar rats administered 10 mg/kg of DMN at two days of age some centrilobular necrosis is produced which is evident histologically over the first thirty-eight hours. The extent and distribution of these lesions, however, is very uneven throughout the liver. At this dose level, from the third day of the experiment onwards, very little change is seen in the liver apart from increased variability in nuclear size. At 20 mg DMN/kg body weight in the Ash-Wistar neonate rat, diffuse necrosis occurs throughout the liver and it would seem reasonable to suppose that the damage in this organ is related to the lethal effects of the compound. This necrosis should be compared to the effect of an equal dose of 20 mg DMN/kg body weight administered to the Carworth/Wistar rats in which strain as reported in the pilot study, no parenchymal cell necrosis was seen and survival was almost one hundred percent.

The liver changes in the Ash-Wistar adult rat seen twenty-four hours after injection of 30 mg DMN/kg body weight were essentially similar to those described by Barnes and Magee (1954) with sharp demarcation between cells that seem to be totally destroyed in the centrilobular region and those which appear to be relatively unaffected in the mid-zonal and periportal zones of the liver lobule. This lesion in the adult was very different from that seen in the two-day old Ash-Wistar rat administered 20 mg/kg where, even at this lower level of

administration, almost complete destruction of the entire liver lobule took place. Decreasing the dose administered to the two-day old rat yet further, to 10 mg DMN/kg body weight, still evoked necrosis, but of a very uneven nature, throughout the liver. That this last dosing regimen induced more pronounced necrosis in the region of the hilus of the liver was confirmed by administering DMN at this level to four additional two-day old rats and killing them 24 hours later. Particular care was taken to take sagittal sections of each lobe radiating from the hilus of the organ. The preferential location of the necrotic areas in this region was then clearly seen.

On this evidence, it is concluded that there is a marked difference between the nature and extent of the lesions induced by DMN in the adult rat liver as compared to the neonate liver. Perhaps it would be more surprising if this was not the case since there are, as discussed below, very great differences in the anatomical structure and metabolising potential of the liver of the neonate when compared to that of the adult rat.

The ability of the liver to metabolise DMN is an important factor in the toxicity of the compound, as has been shown by a number of previous workers. Specifically, early in vitro studies have shown that in a fractionated homogenate of liver the metabolic potential for DMN resides in the microsome plus cell sap portion (Magee and Vandekar 1958; Brouwers and Emmelot, 1960). This metabolic system is the one involved in the hydroxylation and dealkylation of many drugs and foreign compounds including carbon tetrachloride (Magee, 1964; McLean and McLean, 1969). That the acute toxicity of dimethylnitrosamine in the liver is

dependent on metabolism of the compound has been indicated by the protection afforded by cysteine (Emmelot and Mizrahi, 1961) and aminoacetonitrile (Fiume, 1970) both of which inhibit liver demethylating enzymes in vivo. Such a concept is also supported by the work of Heath (1961; 1962) which demonstrated that the relative toxicity of various dialkylnitrosamines correlated with the ability of the liver to metabolise them.

Most biochemical studies with dimethylnitrosamine have been carried out using the adult rat, but expiration of [^{14}C] carbon dioxide following administration of [^{14}C] labelled dimethylnitrosamine to rats less than 24 hours old has demonstrated that this species is capable of metabolising the compound at this age (Terracini and Magee, 1964). Moreover, other experiments have shown that the level of methylation of RNA in the liver increases from birth to reach adult levels by three days of age in Wistar rats (Lee and Spencer, 1964). If it is accepted that methylation of RNA by DMN requires an intermediate alkylating agent, formed from the metabolism of the compound, then it seems reasonable to argue that a gradual development of the metabolising potential of the liver for DMN takes place over the first few days of life.

This suggestion must also be considered in the light of studies which have demonstrated a development of drug-metabolism enzymes in the microsomal fraction of the liver from birth onwards (Jondorf et al., 1958; Fouts and Adamson, 1959; Kato et al., 1964)

With this knowledge of the development of life of processing enzymes over the first few days and the evidence that DMN requires metabolism

to exert its acute hepatotoxic effects, it becomes possible to suggest reasons for the histological observations of this chapter.

In the adult rat a uniform centrilobular necrosis is induced throughout the liver. This may be ascribed to the fact that it is the centrilobular cells which are the more susceptible to the toxic action of DMN because of preferential metabolism in this site.

In the neonate rat however it would seem that it is the hepatocytes in the region of the hilus which possess the greater potential for metabolism of the compound at this age. Thus at a dose level of 10 mg DMN/kg body weight, it is the centrilobular zones in this area which are primarily affected by the treatment. Hepatocytes in the remaining areas of the liver possess some ability to metabolise the toxin, and at a higher dose level of 20 mg/kg diffuse necrosis is induced throughout the organ. It is interesting in this respect that another substance, aflatoxin, has also been shown to bring about diffuse non-zonal necrosis in the liver of the newborn rat whilst the lesion was found to be exclusively periportal in location in the adult (Newberne and Butler, 1969).

The considerable difference in susceptibility to DMN exhibited by the Carworth/Wistar and the Ash-Wistar strains of neonate rats is also worthy of comment. In this connection other workers have reported an increased susceptibility of Porton and Carworth/Wistar adult rats to the lethal effects of DMN over a period of two years although the nature of this change was not understood (McLean and Verschuuren, 1969). The difference between the two strains seen in the work of this chapter

might have been due to the differential rates of development of the microsomal processing enzymes responsible for DMN metabolism in the Ash-Wistar as compared to the Carworth/Wistar rats. Alternatively, it might have reflected a genetically based metabolic difference in susceptibility to DMN in which case a difference in such susceptibility between the two strains might also be demonstrable in adulthood.

The above discussion presents a picture which is overwhelmingly in favour of the acute toxicity of DMN being due to an active intermediate metabolite. A fairly recent report (McLean and Vershuuren, 1969) suggested that neither the rate nor the amount of DMN metabolised is the predominant factor in liver damage. This postulate was based on the fact that dietary depletion protected against the toxic effect of DMN, but this protection, unlike the situation found with carbon tetrachloride, was not reversed by concomitant administration of phenobarbitone or DDT. Both of these compounds have been found to increase the level of drug-metabolising enzymes in the microsomal fractions of the liver in vivo (McLean and McLean, 1966). It was further suggested that DMN, after conversion to a toxic metabolite such as a carbonium ion, attacks cell sites which become accessible or are protected depending on the previous diet. Such an explanation of acute DMN toxicity would fit with the experimental results of this chapter in that these cell sites could be more exposed to attack by the DMN metabolite in the liver of the neonate rat as compared to the adult. This would then explain the much greater susceptibility of the neonate liver to the action of DMN. Certainly, enormous structural develop-

ment in lipoprotein membranes takes place over the first few days of life of the rat (Dawkins, 1959). The alternative explanation of the results of the work of McLean and Verschuuren (1969) is that DDT and phenobarbitone do not induce the enzymes responsible for DMN metabolism. This view was upheld by an investigation into the effect of microsomal enzyme inducing agents on DMN carcinogenesis (McLean and Magee, 1970). In this paper it was reported that DDT and phenobarbitone failed to reverse the protection afforded by dietary depletion, whereas benzo-(a) pyrene administration achieved this effect. Furthermore, DDT and phenobarbitone failed to alter the carcinogenic response for the kidneys of rats given DMN by single injection while on a protein depleted diet, which would have been expected if DDT and phenobarbitone had induced the microsomal enzymes responsible for DMN metabolism. Thus, the more rational postulate to date is still that the extent of the acute effect of DMN in the adult rat is dependent on the amount of the active metabolite of the compound formed, and that dietary procedures do not in themselves expose or protect particular cell sites against its necrotoxic action. Whether or not cell sites are more exposed to DMN in the neonate rat remains an open question although it would still seem that metabolism of the compound is very crucial to its action at this age.

One again, gross depletion of the haemopoietic tissue in the neonate liver was noted over the first two days following injection. Regeneration was seen to be taking place by the third day and was complete by the sixth day. The nature of the effect of DMN on this

haemopoietic tissue in various haemopoietic centres in the rat is explored in the next experiment and will not be discussed further at this point. Suffice to say that this next study lent yet further support to the view that DMN exerts its necrotoxic effect through an active metabolite.

The two experiments reported above, investigating the lethal and histological effect of DMN to the two-day old rat, showed that 10 mg/kg body weight was a suitable dose level for subsequent experimentation.

Experiment 6.4: An investigation of the nature of the necrosis of haemopoietic tissue in the liver following administration of DMN

It was observed in the previous experiment that there was a profound depletion of haemopoietic tissue in the liver following administration of dimethylnitrosamine although no such marked effect was noted in the haemopoietic tissue of the spleen. It therefore appeared from this that the depletion of haemopoietic tissue was mainly localised in the liver. It was felt that confirmation of a localised effect should be obtained by investigating the effect of the poison on an area exclusively concerned with haemopoiesis i.e. bone marrow. The bone marrow of the femur of the neonate rat is an active site of haemopoiesis and as little ossification has taken place at this early age it was realised that histological sections of whole femurs showing haemopoietic tissue in the medullary cavity would not be difficult to obtain. This proved to be the case.

Method

Two-day old Ash-Wistar rats were divided into three groups of six rats per group. One group received 20 μ l physiological saline and the

remaining two groups 10 mg and 20 mg DMN/kg body weight respectively by intraperitoneal injection. Two animals from each group were to be killed at seven, 24 and 48 hours following injection. The liver, spleen, thymus and femurs were removed from each animal and treated in the following manner.

Portions of liver, spleen and thymus were fixed in ten percent neutral buffered formalin, routinely embedded in paraffin wax, sections cut at 4 μ and stained by Ehrlichs alum haematoxylin and eosin and also methyl-green pyronin.

Sections, following dewaxing and hydration were stained for 10 minutes in methyl green pyronin, made up with the following constituents.

200 mls acetate buffer pH 4.8

200 mls dist. aqua

4.8 mls 5% aq. pyronin y

7.2 mls 2% aq. methyl-green.

The buffer was made up as follows

A. Glacial acetic acid 5.3 ml made up to 500 ml with dist. aqua

B. Anhy. sodium acetate 6.15 gm made up to 500 ml with dist. aqua.

A ratio of A:B of 40:60 gave a buffer solution of pH 4.8.

The sections were then rinsed in dist. aqua for ten seconds and drained and dipped in two changes of acetone for two further periods of ten seconds. This was followed by a 20 second treatment in a solution made up of equal parts acetone and xylol. The sections were then finally mounted in DPX.

The processing of the femurs of the neonate rats was carried out as follows.

The whole bones were fixed in neutral buffered formalin for seven days and then decalcified in Gooding and Stewart's fluid (Formic acid - 500 ml, 40% formaldehyde - 100 ml, dist. aqua - 1500 ml) for a further five days. They were then passed through 70 percent ethanol for twenty-four hours, 80 percent and 90 percent ethanol for three hours respectively and then into absolute alcohol for a further 12 hours. They were next transferred to amyl acetate for three hours and passed through two changes of 1 percent celloidin in methyl benzoate over the following forty-eight hours. This was followed by emersion in xylene for one hour and then routine embedding in paraffin wax. The blocks from the above treatment were trimmed carefully so that 4 μ thick sections contained adequate longitudinal sections of the haemopoietic tissue in the medullary cavity. Sections were stained by Ehrlich's alum haematoxylin and eosin.

[It was found that although very little ossification had taken place in the femur by three days of age, minute spicules of calcification tended to drag through the section on cutting the block if decalcification was not carried out. Double embedding was similarly found to be advantageous as the haemopoietic tissue tended to fragment on sectioning and was lost in some areas if this process was not employed.]

Results

i) Survival

Two of the six rats which received 20 mg DMN/kg were killed at seven hours. Three of this group were dead by 24 hours and the lone survivor was killed at this time. The remaining control and DMN dosed

animals survived their treatment for their respective stipulated periods.

ii) Macroscopic findings

No abnormalities were found macroscopically in any animal apart from those which received the dose level of 20 mg DMN/kg. These animals presented the same picture as described in the previous experiment with somewhat swollen and congested livers.

Histological findings

1. Liver

a) Liver parenchyma

The changes observed at 24 hours were essentially as described in the previous section. Irregular areas of severe degenerative change of hepatocytes were seen in the livers from the animals dosed with 10 mg/kg DMN, but diffuse necrosis was observed in those dosed with 20 mg/kg. The livers of the animals dosed with 10 and 20 mg DMN/kg body weight and killed after seven hours did not show any signs of necrosis of hepatocytes.

In the livers of the DMN treated animals killed two days after the initial injection there were irregular areas of coagulative necrosis. Usually these had an essentially centrilobular location but in one instance the entire extremity of one lobe was involved.

b) Haemopoietic tissue

The control livers showed marked haemopoietic activity at all the time intervals studied.

By seven hours necrosis was widespread in the haemopoietic cells

in the livers of animals dosed at both levels of DMN. Degenerating nuclei were most easily seen in the blast cell types with pyknosis and karyorrhexis. It was extremely difficult to be sure of pyknotic nuclei in normoblasts as they usually have very small darkly-staining nuclei. Some islets of haemopoietic cells showed one or two remaining viable cells surrounded by cell and nuclear debris (Fig. 6.6). The megakaryocytes were also altered by the treatment. The nuclei in many cases had become moderately pyknotic and the cytoplasm extremely shrunken and granular with a deep purple colouration.

At the 24 hour interval, at both dosage levels, necrotic haemopoietic cells were still to be seen and there was overall depletion by this time. All cell types in the haemopoietic series were involved in this depletion. Occasionally very pyknotic nuclei of megakaryocytes were encountered (Fig. 6.7). In contrast megakaryocytes in livers from the control animals had large vesicular meganuclei surrounded by bright pink homogenous cytoplasm.

At 48 hours the livers of the animals which had been administered 10 mg DMN/kg body weight were almost completely devoid of haemopoietic tissue.

2. Spleen

The appearance of the spleens from the control animals at all time intervals was one of a contracted red pulp in which there was marked haemopoiesis and which surrounded the large perivascular lymphoid sheaths. Presumably the contraction was related to exsanguination following decapitation.

No abnormalities were seen in the spleens of the animals dosed

with 10 mg/kg DMN. Similarly, the spleens of animals dosed with 20 mg/kg and killed after seven hours presented the same histological picture as seen in the control spleens.

The spleens of the animals dosed with 20 mg/kg DMN, which died within the next twenty-four hours, showed slight engorgement of the red-pulp. In addition, occasional haemopoietic cell nuclei could be observed in karyorrhexis. No such degenerative changes were seen in the animal which survived the dose level of 20 mg/kg and it was concluded that this effect was due to post mortem autolysis.

3. Thymus

No abnormalities were noted in this organ.

4. Bone marrow

The bone marrow of the femurs of both control and dosed animals at all time intervals studied showed marked haemopoietic activity. There was no evidence of necrosis or depletion of the haemopoietic cells at either dose level of DMN. The megakaryocytes were normal in appearance in the bone marrow in contrast to their shrunken appearance in the liver of the same DMN treated animal (Figs. 6.7. and 6.8.)

Discussion

The above descriptions clearly show that following a dose of DMN below the level required to produce extensive necrosis of hepatocytes, gross necrosis of the haemopoietic tissue in the neonate liver is a consistent finding. Haemopoietic tissue sited in bone marrow and spleen would appear to be unaffected by any level of DMN administration.

Until recently the effect of dimethylnitrosamine on haemopoietic tissue has not received much attention in past publications concerning

the toxicity of the compound. Interestingly, Jacobsen et al. (1955) demonstrated a profound drop in circulating leucocytes in dogs within an hour of being allowed to inhale DMN vapour. This leucopaenia showed a relative concurrent increase in the percentage of polymorphonuclear neutrophils. By 24 hours a pronounced leucocytosis had developed. These workers attributed the initial fall in white blood cells to the direct effect of the poison on circulating leucocytes. The detailed differential blood cell figures are not given in this publication.

More recently Chernozemski and Warwick (1970) noted the disappearance of haemopoietic cells from the liver following a single dose of 10 µg DMN/gram body weight to 7-9 day old mice.

The relative differential effect of DMN at the various sites of haemopoiesis must be examined in the light of the distribution of the compound throughout the body following administration. Magee (1956) demonstrated unequivocally that DMN is rapidly and evenly distributed throughout the body water and tissues following administration. Moreover, the excretion of unchanged DMN was at such a low level that it was concluded that the compound was rapidly metabolised; experiments with hepatectomised rats showed that the liver was the major site of this metabolic activity.

Almost total depletion of haemopoietic tissue occurs in the neonate liver, and none at all in the spleen or bone marrow. This suggests that the necrosis observed in the liver is due to a high level of toxic metabolite which is released in this organ and which brings about degeneration of the blood forming cells over the following two days. (The experiment previous to this one had shown that three days following

injection of DMN the haemopoietic tissue had commenced to regenerate). With the even distribution of DMN in the body water (Magee, 1956) the haemopoietic tissue in the spleen and bone marrow would have shown necrosis of an equal magnitude to that seen in the liver, had the effect of the compound been of a direct nature. From the results with the 10 mg DMN/kg rats it appears that although haemopoietic cells are incapable of metabolising DMN to any significant degree, they are in fact more susceptible to the necrotoxic effect of the metabolite than are the liver cells themselves. Presumably any toxic metabolite which leaves the liver via the hepatic venous system is considerably diluted since even at a dose level of 20 mg/kg no necrosis is seen in the haemopoietic tissue of the bone marrow or spleen.

The nature of the toxic metabolite, which has been suggested above to reach high enough levels in the liver to induce necrosis of haemopoietic tissue, is open to debate. The fact that the toxin is able to 'leak' from hepatocytes, in which cell it is formed, indicates that it is not a particularly reactive one. Thus it would seem reasonable to discount carbonium ions from this effect on the blood-forming cells since such toxins would rapidly react with many susceptible cell constituents within the hepatocyte and would be unlikely to gain access to the intercellular fluid in a reactive form.

It could be argued that it is the necrosis of hepatocytes themselves which might be unfavourable to the existence of haemopoietic tissue in the liver. This seems unlikely in view of the fact that carbon tetrachloride, which induced necrosis (see Chapters 7 and 9), did not affect the level of haemopoiesis in the neonate liver.

Illustrations

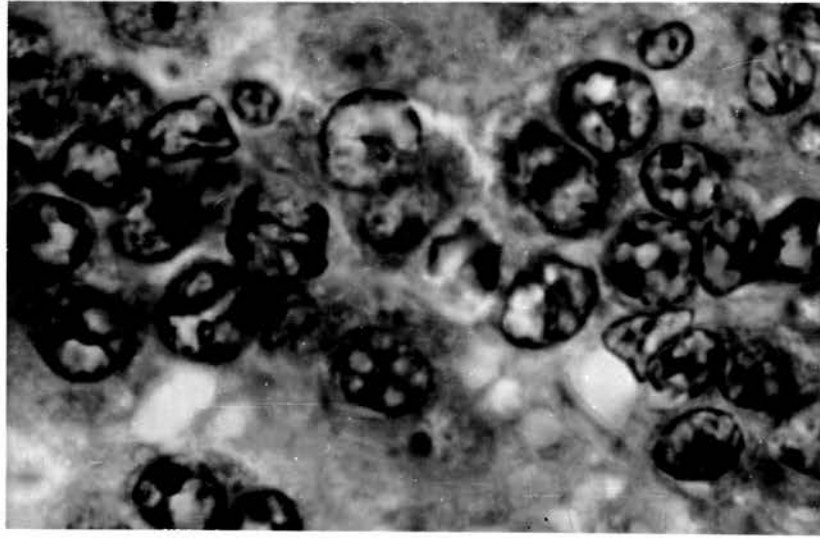


Fig. 6.1 Example of a group of cells found in the liver of a rat injected six days previously with 20 mg DMN/kg body weight. They were thought to be of the lymphocytic series (H+E x 2200).

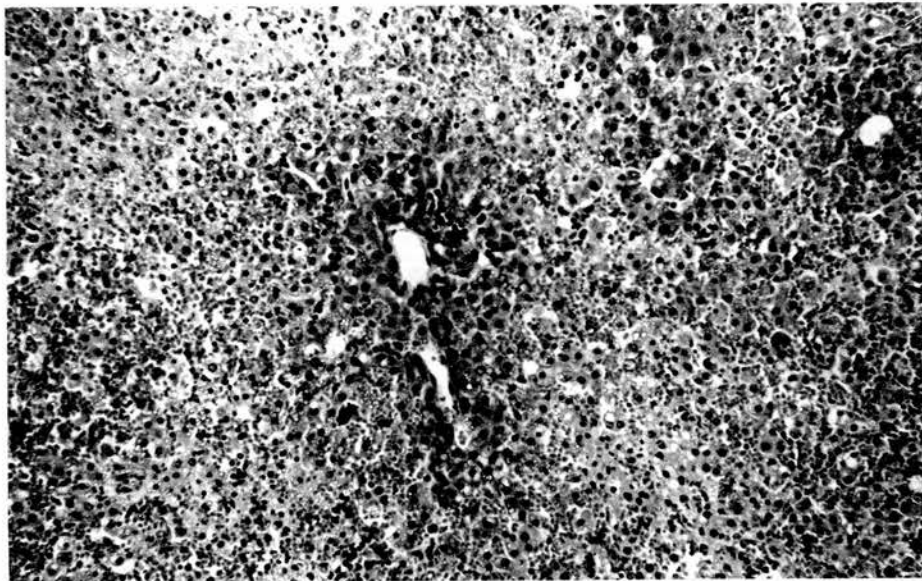


Fig. 6.2 Liver of three-day old rat treated twenty-four hours previously with 20 mg DMN/kg body weight illustrating the massive diffuse necrosis induced at this level of administration (H+E x 220).

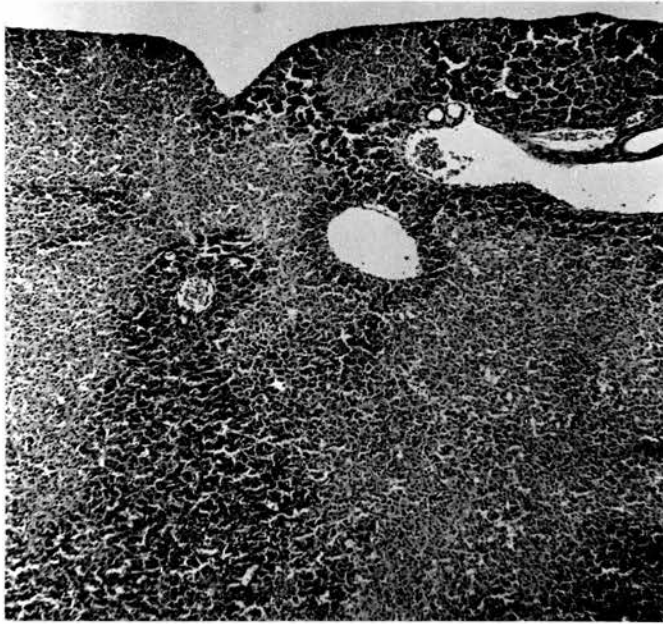


Fig. 6.3 Liver of rat treated twenty-four hours previously with 10 mg DMN/kg body weight and showing large irregular confluent areas of necrosis in the region of the hilus of the organ (H+E x 90).

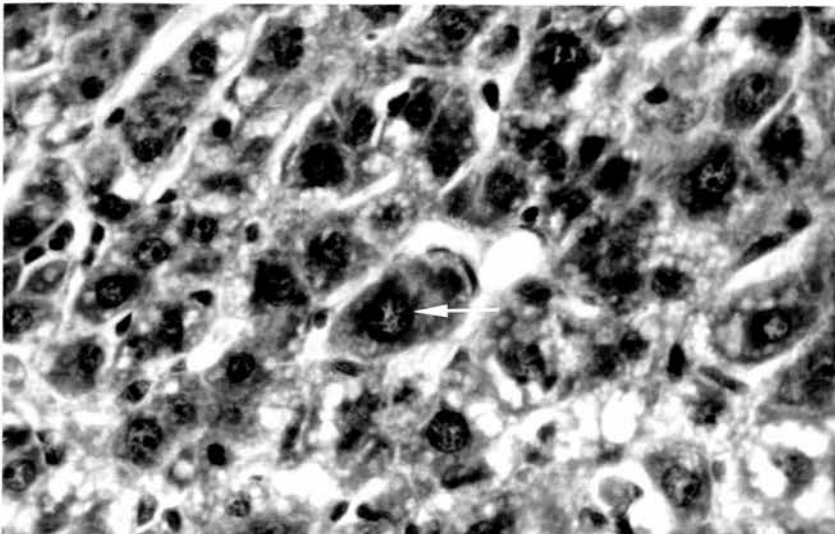


Fig. 6.4 Liver of rat treated fourteen days previously with 10 mg DMN/kg body weight. An enlarged hepatocyte nucleus is seen arrowed (H+E x 360).

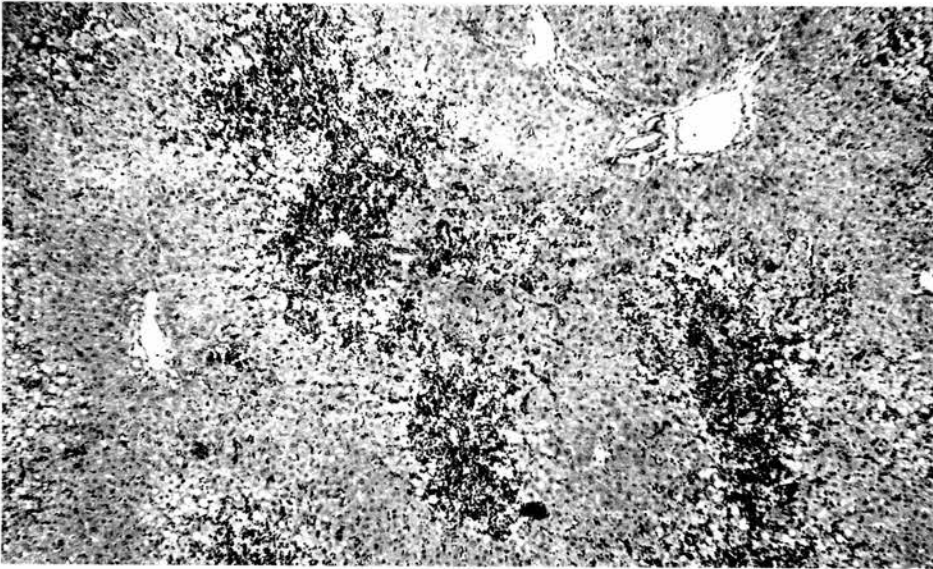


Fig. 6.5 Liver of an adult rat which received 30 mg DMN/kg twenty-four hours previously. Large areas of necrosis and congestion are observable surrounded by vacuolated hepatocytes (H+E x 90).

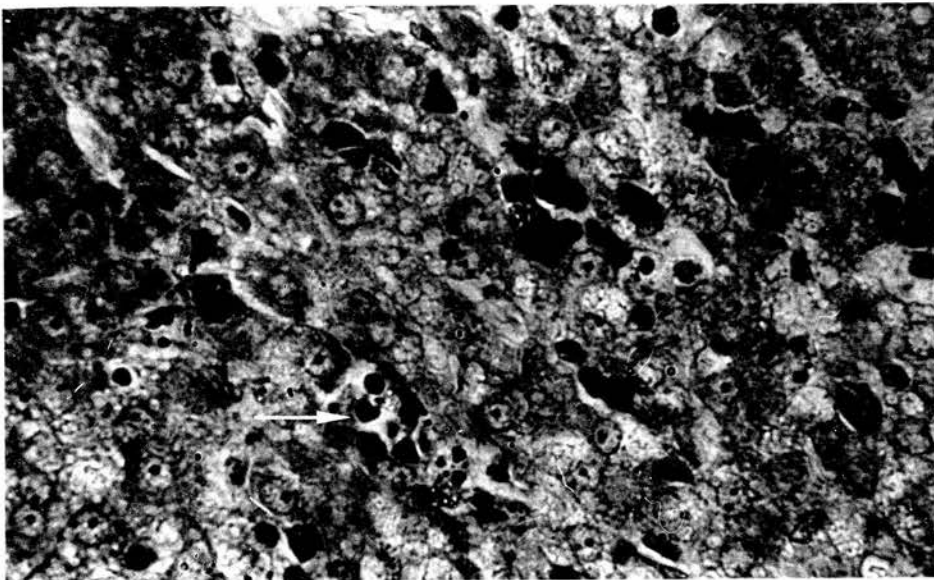


Fig. 6.6 Liver of neonate rat injected with 10 mg DMN/kg body weight twenty-four hours previously. A group of haemopoietic cells with karyorrhetic nuclei is shown arrowed. (Methyl-green-pyronin x 880).

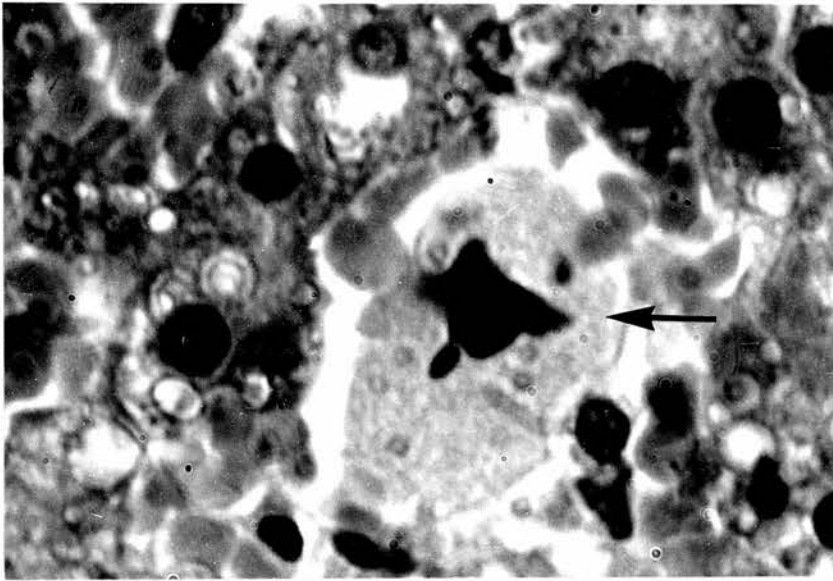


Fig. 6.7 Liver of neonate rat treated with 10 mg DMN/kg body weight twenty-four hours previously. A pyknotic megakaryocyte is seen arrowed (H+E x 2200).

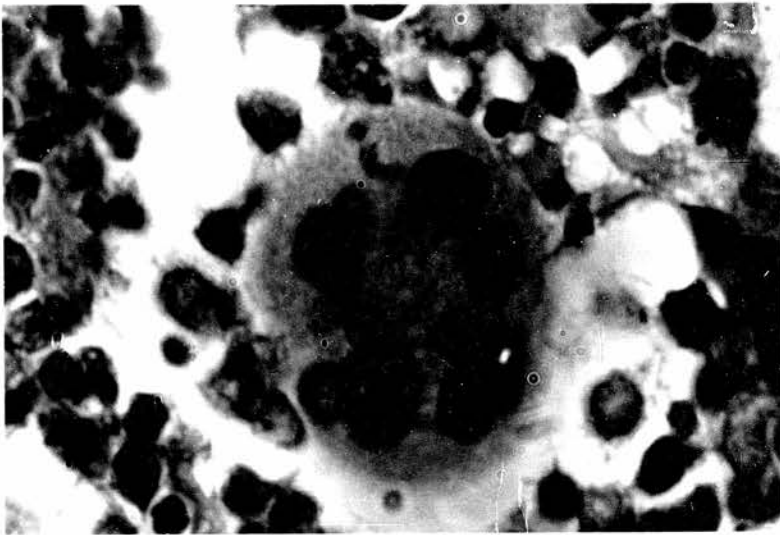


Fig. 6.8 Bone marrow from same rat as in Fig. 6.7 showing completely normal megakaryocyte (H+E x 2200).

CHAPTER 7

PRELIMINARY EXPERIMENTS WITH CARBON

TETRACHLORIDE IN THE NEONATE RAT

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Previous work which has involved the administration of carbon tetrachloride to neonate rats has suggested that the toxicity of the compound varies considerably over the first few days of life (Cameron and Karuraratne, 1936; Dawkins, 1963). The purpose of using carbon tetrachloride in the work of this thesis was to investigate the effect of its necrotoxic action on the ploidy state of the liver. It was therefore essential that the compound be administered to the neonate rat at an age when a uniform necrotoxic response might be expected.

The first experiment described in this chapter is concerned with the variation of necrotoxicity of carbon tetrachloride over the first five days of life and established the earliest age at which the compound could be given in order to obtain the least variation. The second experiment established the LD_{50} of carbon tetrachloride at this age so that a near maximum dose compatible with one hundred percent survival could be used. The third experiment in this series briefly investigated the development and repair of the lesion in the liver at this dose level.

Experiment 7.1

The necrotoxic action of carbon tetrachloride in the liver of the neonate rat during the first five days of life.

Method

Litters of Ash-Wistar rats were taken and the individuals divided into five groups of four rats each so that litters and sexes were, as nearly as possible, equally divided between groups. Group one were treated with 0.1 ml CCl_4 /kg body weight intraperitoneally on the first day of life, group two on the second day of life and so on until groups

were obtained containing rats of age 1-5 days inclusively. Similar control groups of two rats each received 20 μ l physiological saline intraperitoneally. When the animals had been killed by decapitation the liver, kidneys, spleen, heart, lungs, stomach, large and small intestine were removed for histology. The tissues were fixed in ten percent neutral buffered formalin, embedded in paraffin wax and, after sectioning at 4 μ were stained with Ehrlich's alum haematoxylin and eosin.

Results

On macroscopic examination the tissues from both dosed and control animals appeared normal.

No histological abnormalities were found in any of the tissues examined apart from the livers taken from the animals dosed with carbon tetrachloride. [The histological appearance of the livers removed from the control animals were as described in the previous chapter p 136].

Table 7.1 shows the extent of the lesions in the individual rat livers in this experiment.

Table 7.1

Scoring of degenerative changes in the livers of rats
administered carbon tetrachloride at 1-5 days of age

Age in days	Dosed carbon tetrachloride		Control	
	M	F	M	F
1	-	+	-	-
	-	+		
2	+	+	-	-
	-	+++		
3	++	++	-	-
	+++	++		
4	++++	++++	-	-
	++++	++++		
5	++++	++++	-	-
	++++	++++		

- Normal

+ One or two ballooned cells associated with occasional central veins (Fig. 7.3)

++ One or two ballooned cells in centrilobular areas irregularly scattered throughout liver

+++ Moderate number of ballooned cells regularly distributed centrilobularly throughout the liver (Fig. 7.2)

++++ Very marked regular centrilobular degenerative changes and balloon cell formation (Fig. 7.1)

M = Male

F = Female

a) Appraisal of haematoxylin and eosin stained liver sections

i) Livers of animals injected at four and five days of age

All livers of the rats injected at four and five days of age showed pronounced lesions twenty-four hours later. These lesions were associated at all times with hepatic veins and appeared as a zone, the centre of which consisted of numerous hepatocytes undergoing degenerative changes with deeply eosinophilic cytoplasm and pyknotic nuclei. The occasional histiocyte could be seen in this region. Surrounding this zone of necrotic cells was a broad band of ballooned cells which had distended outlines, very little stainable cytoplasm, and markedly pyknotic central nuclei. The hepatocytes in the remaining areas of liver appeared normal as did the portal tracts and haemopoietic tissue. Fig. 7.1 shows the typical appearance of the liver after carbon tetrachloride poisoning at this age.

The animals treated with carbon tetrachloride at an earlier age than four days showed this type of lesion to a varying degree.

ii) Livers of animals injected at three days of age

These animals showed livers with similar but much less pronounced lesions (see Fig. 7.2), the distribution of which was less frequent and more irregular throughout the liver when compared with the four and five day age groups. Many central veins could be identified with no associated degeneration or one or two ballooned cells only. The livers of animals injected up to three days of age showed more pronounced lesions in the region of the hilus of the liver.

iii) Livers of animals injected at two days of age

The areas of degeneration and ballooned cell formation in the livers of the animals dosed at two days of age varied markedly in extent. As may be seen from Table 7.1 one liver showed no lesions whatsoever.

iv) Livers of animals injected at one day of age

The livers of the animals dosed at one day of age again showed variation in the lesions produced by carbon tetrachloride. Two livers exhibited no degenerative changes while the remaining two of this group showed the occasional ballooned cell associated with hepatic veins

Fig. 7.3.

Discussion

It is evident from this experiment that in Ash-Wistar strain rats there is a gradual development of susceptibility of the liver to the necrotoxic action of carbon tetrachloride from birth and this reaches a maximum by four days of age.

There appeared to be no difference between the sexes in their susceptibility to the action of the poison over the period studied.

Dawkins (1963) described the complete insusceptibility of the liver of newborn rats to the hepatotoxic effects of carbon tetrachloride when the compound was administered within 12 hours of birth. In the work of this thesis the earliest age at which the animals were dosed was the day following birth. At this time, two out of the four animals dosed with the chemical showed very slight hepatocellular lesions associated with the 'central' veins. It is possible that, had a group been injected at less than 12 hours of age, they might also have shown complete insusceptibility. Earlier work by Cameron and Karuraratne

(1936) also noted the relative insusceptibility of neonate rat liver to the toxic effects of carbon tetrachloride. In their experiments, subcutaneous injections of 0.1 ml into 6-9g rats (age not stated) were required to produce equivalent lesions to those observed after 0.25 ml was administered to 150g rats.

Dawkins (1963) demonstrated in his own series of experiments that this resistance to toxicity was not due to decreased amounts of carbon tetrachloride reaching the liver. He found that the relative liver concentration following subcutaneous injection in the adult and newborn reached the same order of magnitude, although the highest levels were reached four hours earlier in the case of the newborn animal.

Since the evidence available at the time showed that significant development of lipid-containing membranes took place in the liver of the rat over the first few days from birth (Dawkins, 1959), and that carbon tetrachloride damage in the adult rat liver involved these membranes (Bassi, 1960), Dawkins (1963) postulated that the neonatal resistance to carbon tetrachloride poisoning was due to age differences in the properties and development of these lipo-protein membranes.

Although this non-target effect would satisfactorily agree with suggestions that the action of carbon tetrachloride was due to lipo-peroxidation (Butler, 1961; Recknagel and Ghoshal, 1966) these latter workers also argued that the liver changes required the metabolic formation of a toxin. Butler (1961), who isolated chloroform in small amounts from air expired by dogs administered carbon tetrachloride suggested this might be derived from the release of the free radical CCl_3 . This proposition has been supported by the more recent experiments

of Fowler (1969) in which hexachloroethane was isolated from the tissues of rabbits administered carbon tetrachloride. Fowler concluded that the hexachloroethane could well have arisen from dimerization of trichloromethyl free radicals.

With the compound rapidly distributed throughout other tissues, Recknagel (1967) has argued that the selective damage to the liver parenchymal cell in carbon tetrachloride poisoning does not favour a direct lipid-solvent action of the compound on cell membranes as an explanation of toxicity. Additional evidence with regard to hepatotoxicity depending on the metabolism of carbon tetrachloride has come from experimental manipulations such as a depletion of protein in the diet (McLean and McLean 1969) or concurrent administration of substances such as phenobarbital or DDT (McLean and McLean 1966; Seawright and McLean 1967; Garner and McLean 1969) where alterations in the levels of microsomal drug processing enzymes lead to modification of carbon tetrachloride toxicity in the liver.

These facts concerning the metabolism of carbon tetrachloride assume particular significance when the results of Experiment 7.1 are considered in relation to research which shows that drug metabolising enzymes develop over the first few days of life (Jondorf et al., 1958; Fouts and Adamson, 1959; Kato et al. 1964). On these grounds it seems probable that the resistance of the neonate rat liver to the toxic effects of carbon tetrachloride is a result of the relative inability of this organ to break down the compound to the active metabolite.

This experiment demonstrated that the earliest age at which a uniform necrotoxic effect could be expected to be produced in this

strain of rat was four days. The remainder of the work on carbon tetrachloride in the neonate thus involved administration of the compound to rats of this age.

The next experiments determine the LD₅₀ of CCl₄ in the four day old rat and describe the further development of the lesions produced.

Experiment 7.2

An estimation of the median lethal dose of carbon tetrachloride in male and female rats at four days of age.

Method

Four groups of 4-day old rats were taken. Each group comprising five males and five females was dosed intraperitoneally with either 0.1, 0.2, 0.4 or 0.8 ml/kg carbon tetrachloride. The time of death of any animal was noted and the survivors killed on the seventh day following the initial injection. As has already been described in the DMN LD₅₀ experiment, those animals which were cannibalised were assumed to be mortalities of the carbon tetrachloride treatment. The median lethal dose was calculated according to the method of Weil (1952).

Results

Table 7.2 sets out the number of deaths of animals in any day following the injection of carbon tetrachloride (c = animal cannibalised on that day).

Table 7.2

Survival of rats injected with CCl₄ at 4 days of age

Sex	Dose level CCl ₄ /kg body weight	No. of deaths on any day following injection CCl ₄						Survival at day 7
		Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	
Males	0.1							5
	0.2					1		4
	0.4	3			1	1		0
	0.8	4 ^{cc}		1				0
Females	0.1							5
	0.2					3 ^c		2
	0.4	2 ^c	1			1		1
	0.8	4	1					0

Calculations

(See p 131 for details of formula used).

$$K = 3, \quad n = 5$$

Males

$$\log m = \overline{1}.0000 + 0.301(0.3+1) \pm 2(0.3010)(0.2)$$

$$m = 0.25 \text{ ml/kg} \pm 0.13$$

$$LD_{50} = 0.25 \pm 0.13 \text{ ml/kg carbon tetrachloride}$$

Females

$$\log m = \overline{1}.0000 + 0.3010 (0.1+1) \pm 2(0.3010)(0.3165)$$

$$m = 0.21 \text{ ml/kg} \pm 0.19$$

$$LD_{50} = 0.21 \pm 0.16 \text{ ml/kg carbon tetrachloride}$$

Discussion

Although the values obtained for the LD_{50} 's of males and females respectively, differed by 0.04 ml/kg, in terms of the 95% confidence limits this difference is obviously not significant.

No references concerning the LD_{50} of carbon tetrachloride in rats have been found. However, Cameron and Karuraratne (1936) observed a high level of survival in neonate rats (6-10g/body weight) administered 0.1 ml of carbon tetrachloride. The mean weight of the rats used in Experiment 7.2 above was approximately 10 grams. Thus it would seem that the rats in Cameron and Karuraratne's work were dosed before full susceptibility to the toxic effects of carbon tetrachloride had developed.

The LD_{50} values obtained in the experiment above certainly indicate that once full susceptibility to the chemical has developed the young rat is considerably less able to withstand the toxic effects of carbon tetrachloride. This may be seen from the examples of publications given in Table 7.3, which quote dosage levels of carbon tetrachloride administered to albino rats.

Table 7.3
Reported levels of administration of carbon tetrachloride
to albino rats and subsequent survival

Reference	Age	Weight	Route of Administration	Dose level	Survival
Experiment 6.2 above	4 days	~ 10 gm	i/p	0.23 ml/kg	~ 50%
Cameron and Karuraratne (1) (1936)	Neonate	6-9 gm	s/c	10.0 ml/kg	100%
" (2)	Adult	150 gm	s/c	3 ml/kg	100%
Christie and Judah (1954)	Adult	120-150 gm	Intragastric	2.5 ml/kg	~ 50%
Post et al. (1957)	Adult	150 gm	i/p	0.6 ml/kg	100%

The results of Experiment 7.2 indicated that the highest dose of carbon tetrachloride compatible with one hundred percent survival in the Ash-Wistar strain of rat was 0.1 ml/kg.

The further development of the liver lesion at this dose level is determined and discussed in the next experiment.

Experiment 7.3

To study the development and repair of lesions following the administration of 0.1 ml/kg carbon tetrachloride to 4-day old rats.

Method

Fourteen rats were dosed with 0.1 ml/kg carbon tetrachloride and four rats with 20 µl physiological saline by intraperitoneal injection at four days of age. The sexes were equally distributed within the dosed group. Two male and two female rats from the group dosed with carbon tetrachloride and one rat from the control group were killed at daily intervals for four days following the initial injection.

The tissues were removed, processed and stained with Ehrlich's alum haematoxylin and eosin. Appropriate sections were examined for lipid by the Oil red O technique, for reticulin by the method of James, and for collagen by Van Geison's method.

Results

No abnormalities were discernible on macroscopic examination. Histologically, abnormalities were seen in only the livers of the animals dosed with carbon tetrachloride.

a) Liver

(i) Day 1

The lesions in the liver at this time were extensive and essentially as described in Experiment 7.1. There were large areas of ballooned cells surrounding areas of degenerative hepatocytes and cell debris associated with hepatic veins. A few histiocytes were observed in the centre of these lesions. The remaining hepatocytes in intact areas of the liver appeared normal although mitotic activity of the parenchymal cells seemed to be increased to some degree. The portal tracts and haemopoietic tissue were unaffected by the treatment.

The sections stained for lipid showed an indication of slight increase in this constituent over that of the control liver, the hepatocytes containing relatively larger droplets in areas of liver other than the necrotic zone.

The sections stained for reticulin showed that the reticulin framework remained intact in all but the zone of the lobule where ballooned cells had formed. The reticulin fibres in this area gave the impression of being pushed aside by the gross distention of these cells.

(ii) Day 2

The evidence of necrosis had diminished by this time and only a small number of ballooned and necrotic cells were associated with hepatic veins. Mitoses of the parenchymal cells were still relatively frequent.

The lipid content of the livers of the carbon tetrachloride dosed animals had increased greatly by this time as compared to the control

liver and hepatocytes in all zones of the lobule showed large droplets of lipid material.

(iii) Day 3

The repair of the liver necrosis in terms of cell replacement was complete by this time though the haematoxylin and eosin sections showed increased vacuolation of hepatocytes in all areas of the lobule.

The lipid content of the livers of the dosed group was still as high as at day 2.

(iv) Day 4

The only abnormality noticed at this time interval was the slight elevation of lipid content in the CCl_4 dosed group but the level was well below that observed on day 3. The liver of the dosed animals appeared normal in every other respect.

Sections stained by the Van Geison method failed to reveal an increase of fibrous tissue in any location in the liver lobule over the four days of the experiment.

Similarly, the sections stained specifically for reticulin showed a completely normal supporting framework for the liver lobule by day four of the experiment.

Discussion

The acute lesion developed rapidly in the four-day old rats dosed with CCl_4 and was most severe after twenty-four hours. By forty-eight hours, this lesion had regressed considerably and restoration was completely effected within a further twenty-four hours.

In contrast to the necrotoxic changes, the increase in lipid

content seen in the animals dosed with carbon tetrachloride was not markedly different from that of the control livers twenty-four hours after injection. By forty-eight hours, lipid content was increased and this remained at high levels for at least a further twenty-four hours. The lipid content of the livers of carbon tetrachloride dosed animals had however decreased considerably by the fourth day.

The ballooned cells did not show any lipid content. This finding is in agreement with other workers who have ascribed this change to one of hydropic degeneration (Cameron and Karuraratne, 1936; Christie and Judah, 1954; Wigglesworth, 1964).

Lipid increases following carbon tetrachloride administration as measured by biochemical techniques is rapid. Significant increases in triglycerides have been recorded as little as one hour after injection (Schotz and Recknagel, 1960; Rees and Shotlander, 1963; Lombardi and Ugazio, 1965). In contrast to this, histological techniques, used to monitor this lipid increase, are less sensitive and results obtained would appear to depend on the technique used to demonstrate the fat. Using a Sudan III frozen section technique, Cameron and Karuraratne (1936) found only slight increases in the lipid content of livers of carbon tetrachloride dosed animals at twenty-four hours as compared to controls, but very significant increases by two to three days after administration. On the other hand, Wigglesworth (1964) found marked lipid increases at eighteen to twenty-four hours as demonstrated with an ethyl-osmium-gallate technique. Thus, it would not seem valid to compare the rate of development of lipid accumulation as seen in the

neonate in this work, with observations published on this hepatotoxic effect in the adult.

It is not intended at this stage to discuss in detail the healing process of neonate liver following carbon tetrachloride poisoning as this is best left to later experimental discussion on the actual mitotic activity of the parenchymal cells involved in the regenerative process. It is sufficient to mention at this point that healing in the young rat has been shown to be considerably more rapid than in the adult following carbon tetrachloride administration (Post et al. 1957; 1960). Healing, as observed by Cameron and Karuraratne (1936), results in complete regeneration and restoration of the liver, with no evidence of replacement repair by fibrosis.

The three experiments described in this chapter failed to demonstrate any sex difference in the rate of development of susceptibility with age. Nor has survival, or extent of development and repair of the liver lesion following carbon tetrachloride administration, been seen to be influenced by the sex of the neonate rat. In contrast the adult female rat has been shown to be more susceptible to the acute action of carbon tetrachloride as determined by glutamic pyruvic transaminase and the lipid content of the liver (Bengmark and Olson, 1962). This is somewhat difficult to reconcile with observations following long term administration when female rats were found to show a better survival rate (György et al., 1946) and to develop cirrhosis to a lesser degree (Reddy et al., 1962). These latter facts could perhaps indicate a possible dissociation of the acute and chronic effects of the compound with regard to the influence of sex on the lesions.

As a result of the experiments described in this chapter it was decided, that in future work, a dose level of 0.1 ml/kg CCl₄ would be used in the four-day old rat.

Illustrations

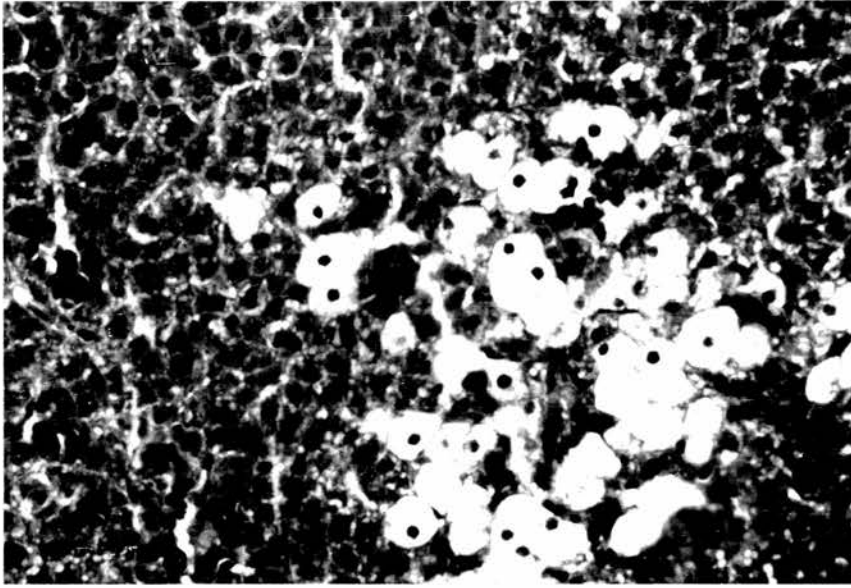


Fig. 7.1 Liver from a 5-day old rat killed 24 hours after an injection of 0.1 ml/kg carbon tetrachloride and showing the extensive zone of the ballooned cells induced by the poison (H+E x 220).

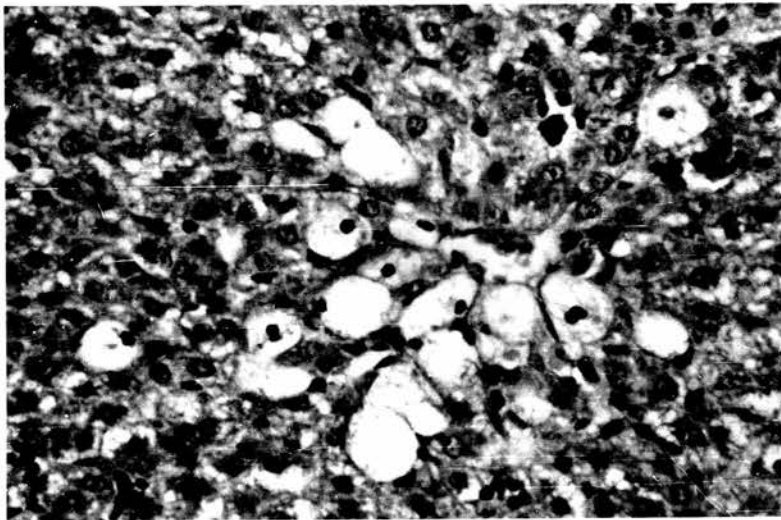


Fig. 7.2 Liver from a rat injected at three days of age with 0.1 ml carbon tetrachloride/kg body weight and killed 24 hours later. The lesion is less extensive than that induced in the 4-day old rat liver as seen in Fig. 7.1 above (H+E x 220).

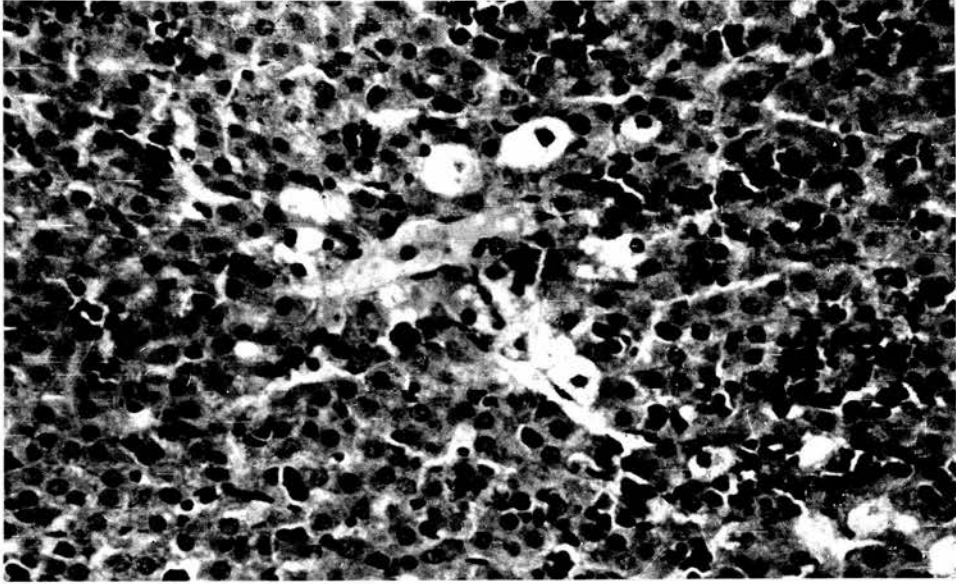


Fig. 7.3 Liver of a rat injected at one day of age with 0.1 carbon tetrachloride/kg body weight and killed 24 hours later. The most extensive lesions seen in this group resembled that shown above with one or two ballooned cells associated with central veins (H+E x 220).

CHAPTER 8

THE NATURE OF NUCLEAR ENLARGEMENT FOLLOWING THE
ADMINISTRATION OF DIMETHYLNITROSAMINE TO THE
2-DAY OLD RAT

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It has been seen that the administration of DMN to the neonate rat results in a significant increase in the size of a nuclei in a proportion of the hepatocytes and that the neonate liver provides an excellent experimental system to investigate the nature of this change.

The experiments in this chapter were carried out to investigate the mechanism of nuclear enlargement and the rate of accumulation of these nuclei following DMN administration.

A survey of published work on physiological and pathological nuclear enlargement indicated that these aims might best be achieved through a quantitative study of nuclear DNA content and synthesis, mitotic activity, and the proportion of binucleate cells of the liver following a single dose of the hepatotoxic chemical.

In the preliminary experiments described in Chapter 6 a suitable dose of DMN was found to be 10 mg/kg body weight.

Experiment 8.1

An investigation of the rate of development of nuclear polyploidy following a single dose of DMN to the 2-day old rat.

Method

Ash-Wistar rats were injected intraperitoneally at 10 a.m. at two days of age with 10 mg dimethylnitrosamine/kg body weight dissolved in physiological saline, or 20 μ l physiological saline by intraperitoneal injection. They were killed at daily intervals for six days and then on the ninth, fourteenth and twenty-first day following the initial injection. The dosing and killing of the animals were so arranged that there were four rats in each group at

each time interval (apart from days 5 and 14 when there were three rats in each group only). As far as possible the sexes and litters were evenly distributed between control and DMN groups.

Following sacrifice, portions of the livers of each animal were disaggregated as described (p 98) and smears prepared and stained by the Feulgen aqueous light green technique. The DNA content was measured in one hundred nuclei in each liver sample. Following computation and assessment of arbitrary DNA units, nuclei were divided into ploidy classes and the means calculated for each group at each time interval.

Results

Table 8.1 sets out the percentage mean of nuclei in each ploidy class found in control and DMN-dosed groups at each time interval (Appendix - Table 3 shows detailed individual observations).

Text-Fig. 8.1 illustrates the increase in nuclear polyploidy in DMN treated and control groups.

Statistical analysis

This was based on the percentage of tetraploid cells in the DMN groups. The hypertetraploid nuclei observed in the treated groups were too few in number for any meaningful test to be carried out.

i) Control v. treated groups

No statistical analysis was carried out as there was an obvious and consistent difference between DMN treated and control groups with regard to nuclear polyploidy.

ii) Treated groups

An analysis of variance carried out on the square roots of individual tetraploid observations in the treated groups revealed a significant variation among the group means:-

$$F = 3.01 \quad n_1 = 8, \quad n_2 = 27 \quad p < 0.025$$

The group means were then examined to determine whether the variation could be expressed as a function of the age or time intervals at which the observations were made. With no prior indication of the form such a function might have, a polynomial regression was used.

It was found that the lowest degree polynomial which accounted for a statistically significant amount of the between group variation was a cubic.

	sum squares	df	mean square	F	p
Due to linear	0.52106	1	0.52106	1.10	-
Due to quadratic	2.64477	2	1.32238	2.80	<0.1
Due to cubic	6.79653	3	2.26551	4.79	<0.01

Thus the best-fit curve for the means of the tetraploid nuclei in the treated groups is in cubic form ($p < 0.01$).

This statistical analysis confirms that the proportions of tetraploid nuclei in the treated groups varied with the time following administration of DMN. The fact the best-fit curve was in cubic form indicated that the tetraploid nuclei reached their maximum numbers over the 4th-6th day of the experiment. From this time onwards their numbers slowly declined over the remaining fifteen days of the experiment.

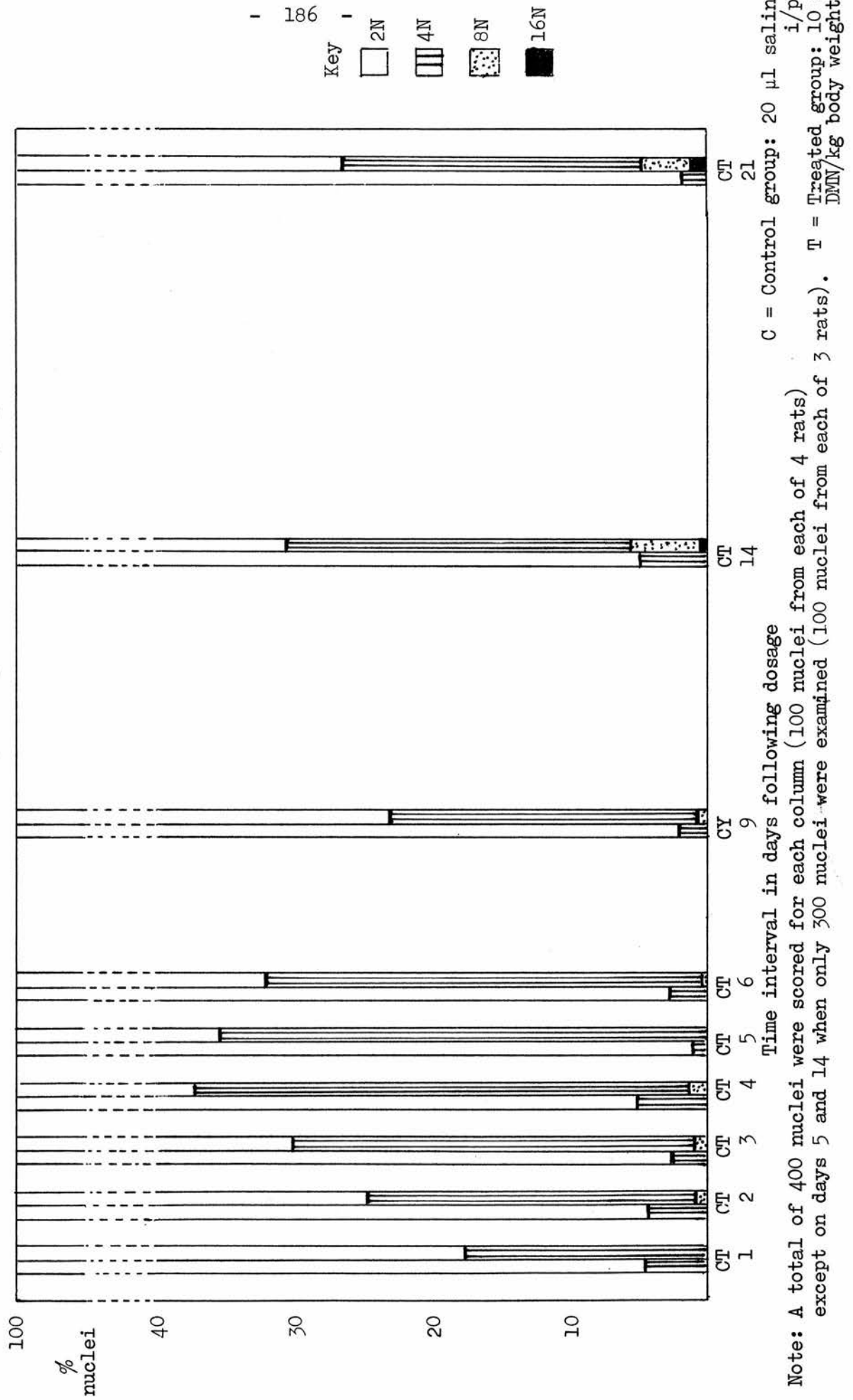
Table 8.1

Percentage means of the hepatocyte nuclei in each ploidy class in groups of Ash-Wistar rats dosed at 2 days of age and killed at varying times thereafter

Time between dosing and death (in days)	Dosage regimen							
	Control group (20 μ l saline i/p)				Treated group (10 mg DMN/kg body weight)			
	(100 nuclei examined in each rat liver)							
	Nuclear ploidy							
	2N	4N \pm S.D	8N	16N	2N	4N \pm S.D	8N	16N
1	95.50	4.50 \pm 2.08	-	-	82.25	17.75 \pm 3.69	-	-
2	95.75	4.25 \pm 1.89	-	-	75.25	24.00 \pm 10.89	0.75	-
3	97.50	2.50 \pm 1.26	-	-	70.00	29.00 \pm 8.02	1.00	-
4	95.00	5.00 \pm 1.61	-	-	62.75	36.00 \pm 5.39	1.25	-
5*	99.00	1.00 \pm 0.77	-	-	64.70	35.30 \pm 0.77	-	-
6	97.25	2.75 \pm 1.26	-	-	68.50	31.25 \pm 10.55	0.25	-
9	98.00	2.00 \pm 2.16	-	-	77.00	22.50 \pm 6.35	0.50	-
14*	95.25	4.75 \pm 2.31	-	-	69.70	25.00 \pm 5.20	5.00	0.30
21	98.25	1.75 \pm 1.26	-	-	73.60	21.80 \pm 4.61	3.40	1.20

Each group contained 4 rats except those marked * which contained 3 rats.

Text-Fig. 8.1 Distribution of the mean percentages of hepatocyte nuclei in each ploidy class in groups of Ash-Wistar rats dosed at 2 days of age and killed at varying times thereafter.



Note: A total of 400 nuclei were scored for each column (100 nuclei from each of 4 rats) except on days 5 and 14 when only 300 nuclei were examined (100 nuclei from each of 3 rats).

Discussion

Following administration of dimethylnitrosamine at two days of age there was a rapid accumulation of tetraploid nuclei so that they reached their maximum numbers by the fourth day of the experiment. The proportion of tetraploid nuclei then declined slowly from approximately 35 percent to approximately 22 percent by day 21. In the control groups the means of tetraploid nuclei formed only 1-5 percent of the observed liver cell population over the three week period studied.

Octoploid nuclei were present as early as the first day following administration of DMN but remained at a low level until the ninth day. By the fourteenth day their numbers had increased considerably. 16N nuclei were not observed until the fourteenth day following the injection of DMN and remained a minor component of the liver cell population over the remainder of the period studied.

No hypertetraploid nuclei were observed in the control liver samples.

Control experiment

As has been set out in materials and methods (p 118), the technique which was used for scanning the smear preparations of liver involved the measurement of only those nuclei which were contained in enough cytoplasm to enable one to positively identify them as hepatocytes.

It was therefore felt necessary to give some indication that the proportions of the various ploidy classes were representative of those actually present in the liver and that the disaggregation technique

did not in itself have a biased effect on the disruption of the cytoplasm of either the diploid or hyperdiploid cells.

The proportions of diploid or hyperdiploid nuclei with or without cytoplasm were therefore estimated after three differing periods of liver disaggregation four days after an injection of 10 mg DMN/kg body weight when the proportion of 4N nuclei, as seen in Experiment 8.1, had reached its maximum.

Method

Four female Ash-Wistar rats were dosed at two days of age with 10 mg DMN per kg body weight by intraperitoneal injection and killed four days later. The livers were disaggregated in a solution of TPB as described and drops of the cell suspension taken five, fifteen and sixty minutes afterwards. Smears were prepared and stained with Feulgen aqueous light green. One hundred nuclei from each liver sample were scored according to their DNA content as diploid or hyperdiploid with or without intact cytoplasm. There were, then, four groups of results for each rat at each time interval.

Results

Table 8.2 shows the percentage of nuclei in each ploidy class obtained from the four animals for each of the three time intervals.

Table 8.2

Nuclear ploidy from intact and disrupted
hepatocytes isolated from rats dosed 4 days
previously with DMN

Rat No.	5 mins.				15 mins.				60 mins.			
	2N Cyt	2N No Cyt	4N Cyt	4N No Cyt	2N Cyt	2N No Cyt	4N Cyt	4N No Cyt	2N Cyt	2N No Cyt	4N Cyt	4N No Cyt
1	34	28	28	10	56	10	30	4	50	8	39	3
2	52	16	24	8	54	13	29	4	56	9	31	4
3	46	22	22	10	50	11	32	7	48	17	26	9
4	39	26	18	17	53	7	31	9	47	13	33	7
Totals	171	92	92	45	213	41	122	24	201	47	129	23
Mean	42.75	23.0	23.0	11.25	53.25	10.25	30.5	6.0	50.25	11.75	32.25	5.75

(4N in this table = hyperdiploid)

A different way of displaying the results may be seen in Table 8.3.

This table shows the percentage of hyperdiploid nuclei devoid of cytoplasm expressed as percentage of the total hyperdiploid nuclei scored for each animal at each time interval.

$$\text{i.e. } \frac{\% \text{ hyperdiploid nuclei with no cytoplasm}}{\% \text{ hyperdiploid nuclei with intact cytoplasm} + \% \text{ hyperdiploid nuclei with no cytoplasm}} \times 100$$

Table 8.3

Percentage hyperdiploid nuclei from disrupted hepatocytes of total hyperdiploid nuclei

Animal	5 mins.	15 mins.	60 mins.
1	26.3	11.7	6.8
2	25.0	12.5	11.4
3	31.3	18.0	25.8
4	48.6	20.9	17.5
Mean	32.8	15.8	15.4

An analysis of variance on these figures (square roots not employed in this instance) failed to reveal a significant difference between animals $F = 4.17$ $n_1 = 3$, $n_2 = 6$ $p > 0.05$

but a significant difference between time intervals

$$F = 11.88 \quad n_1 = 2 \quad n_2 = 6 \quad p < 0.01$$

It is concluded from this analysis that after five minutes of liver cell disaggregation there was a significantly greater proportion of hyperdiploid nuclei with disrupted cytoplasm than at the remaining two time intervals.

The actual scanning technique in other experiments measured only those nuclei with intact cytoplasm. Table 8.4 therefore sets out the percentage of hyperdiploid nuclei with intact cytoplasm expressed as a percentage of all nuclei with intact cytoplasm,

$$\text{i.e. } \frac{\% \text{ hyperdiploid nuclei with intact cytoplasm}}{\% \text{ hyperdiploid nuclei with intact cytoplasm} + \% \text{ diploid nuclei with intact cytoplasm}} \times 100$$

Table 8.4

Percentage of hyperdiploid nuclei with intact cytoplasm

Animal	5 mins.	15 mins.	60 mins.
1	45.1	34.9	43.8
2	31.6	34.9	36.5
3	32.3	38.6	35.1
4	40.0	37.8	41.2
Mean	37.3	36.5	39.2

An analysis of variance on these figures failed to reveal a significant difference between animals $F = 2.38$ $n_1 = 3$ $n_2 = 6$ $p > 0.05$ or between times of disaggregation $F = 0.55$ $n_1 = 2$ $n_2 = 6$ $p > 0.25$.

Discussion

It would seem that although a greater proportion of the cells were disrupted in the initial period of the disaggregation as compared to the later time intervals, the ratio of diploid to hyperdiploid intact cells remained approximately constant throughout and neither class showed a differential fragility.

The high proportion of disrupted cells seen after five minutes was no doubt a reflection of the mechanical damage brought about by the mincing of the tissues with scissors prior to disaggregation.

It will be recalled from Chapter 5 that in the technique used, the cell suspension was discarded after the initial five minutes separation and the actual sample used, collected after a further ten minutes disaggregation.

These results on liver cell disaggregation would seem to indicate that the method used and results obtained in Experiment 8.1 were a true reflection of the proportions of the nuclear ploidy classes present in the livers of the control and DMN dosed animals.

In the first section of the literature review it was seen that mitosis of binucleate cells could contribute significantly towards the formation of polyploid nuclei. The choice of the neonate liver as an experimental system was influenced by the fact that, at this age, there is a negligible proportion of binucleate cells and it was thought that mechanisms of polyploid formation other than binucleate cell mitosis would be more easily evaluated.

Hepatic binuclearity was investigated in neonate liver following DMN administration with a view to confirming that there was no involvement of binucleate cells in the initial rise in polyploid nuclei. The relationship between binucleate cells and the formation of hypertetraploid nuclei which were seen to increase in number up to the end of the three week period studied was also relevant to the design of this experiment.

Experiment 8.2

An investigation of the influence of a single dose of dimethylnitrosamine on the proportion of binucleate cells in the neonate rat.

Method

Ash-Wistar rats were dosed at 10 a.m. on the second day of post-natal life with either 10 mg DMN/kg body weight or 20 μ l physiological saline. They were killed at daily intervals up to the sixth day and then on the ninth, fourteenth and twenty-first day following the initial injection. This procedure was repeated as the litters became available until there were two males and two females represented in

each of the control and DMN dosed groups.

Following sacrifice, the samples of liver were disaggregated as described on p 98 and the suspension of liver cells stained with Trypan Blue. A sample of this cell suspension was counted in a haemocytometer and 2000 cells were scored as mononucleate or binucleate. Binucleate cells in which the nuclei were hyperdiploid were scored separately from binucleate cells with two diploid nuclei (see Fig. 8.1).

Results

Table 8.5 shows the mean and standard deviation of the percentage of binucleate cells in the dosed and control groups. The proportions of binucleate cells at various time intervals may be seen in Text-Fig.

8.2. (Table 4 in the Appendix shows the individual values observed.)

Fig. 8.1 A suspension of hepatocytes in a haemocytometer, stained with Trypan Blue, demonstrating the size criterion which enabled one to class binucleate cells as containing two diploid or two hyperdiploid nuclei. (Liver sample twenty-one days following injection of 10 mg DMN/kg body weight).

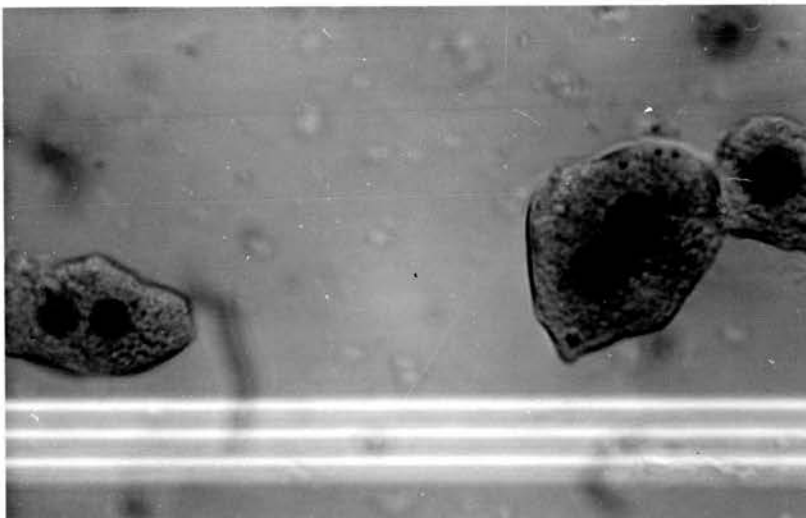
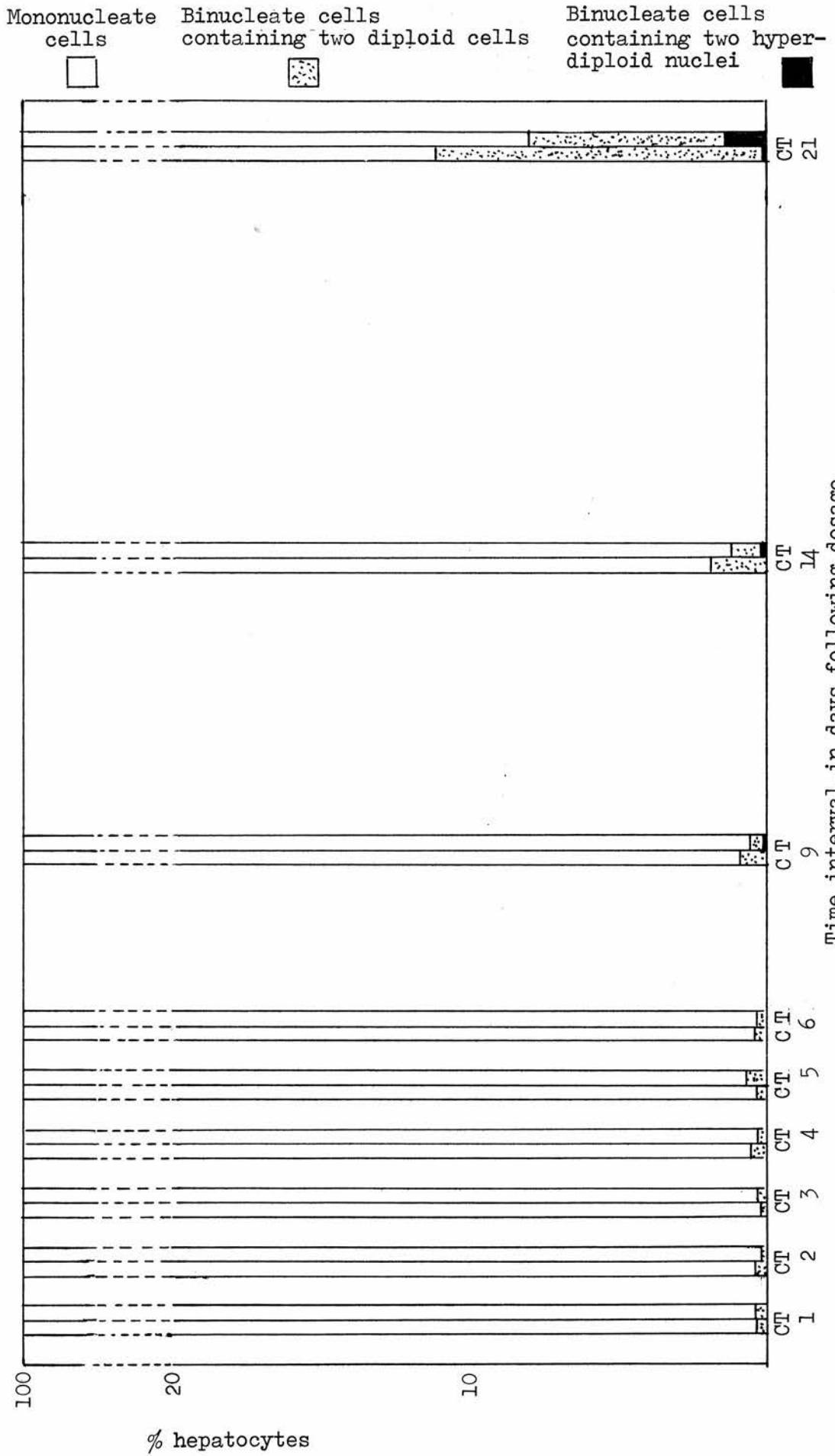


Table 8.5

Percentages of binucleate cells (containing either 2 diploid or 2 hyperdiploid nuclei) in groups of Ash-Wistar rats dosed when 2 days old and killed at varying times thereafter

Time between dosing and death (in days)	Dosage regimen					
	Control group: 20 μ l saline i/p			Treated group: 10 mg DMN/kg body weight		
	% Mononucleate cells	% Binucleate cells \pm SD		% Mononucleate cells	% Binucleate cells \pm SD	
		containing 2 diploid nuclei	containing 2 hyperdiploid nuclei		containing 2 diploid nuclei	containing 2 hyperdiploid nuclei
1	99.65	0.35 \pm 0.14	-	99.55	0.45 \pm 0.20	-
2	99.58	0.44 \pm 0.11	-	99.71	0.29 \pm 0.17	-
3	99.71	0.29 \pm 0.15	-	99.61	0.39 \pm 0.22	-
4	99.40	0.60 \pm 0.33	-	99.62	0.38 \pm 0.18	-
5	99.56	0.34 \pm 0.17	-	99.27	0.71 \pm 0.10	-
6	99.57	0.43 \pm 0.24	-	99.58	0.41 \pm 0.14	-
9	99.02	0.98 \pm 0.22	-	99.50	0.56 \pm 0.23	0.013
14	98.46	1.54 \pm 0.68	-	98.61	1.25 \pm 0.33	0.14 \pm 0.13
21	88.99	11.10 \pm 1.04	0.013	91.98	6.58 \pm 1.78	1.45 \pm 0.41

Text-Fig. 8.2 Distribution of the percentages of mononucleate and binucleate hepatocytes in groups of Ash-Wistar rats dosed when two days old and killed at varying times thereafter.



Note: A total of 8000 hepatocytes were scored for each column (2000 cells from each rat).

Discussion

The proportion of binucleate cells in the control livers remained at very low levels until 11 days of age (day nine following dosage). Their numbers increased slightly by the sixteenth day of age but a sharp rise was seen by 23 days of age when they constituted approximately 11 percent of the hepatocyte population. The nuclei of the binucleate cells in the control livers were almost exclusively diploid in contrast to those from the livers of the DMN treated animals.

This pattern of binucleate cell accumulation seen in the control rat livers is discussed further in Chapter 9 (p 254) with respect to the findings of other workers.

Dimethylnitrosamine treatment had no effect on the proportion of binucleate cells in the liver over the first six days following administration. After this time, the rate of development of these cells in the treated groups paralleled that of the control liver binucleates although within the DMN dosed rat livers the nuclear ploidy of a proportion of these binucleate hepatocytes was found to be hyperdiploid. This was not so in the control livers where the nuclei of binucleate cells were seen to be almost exclusively diploid.

It would seem, therefore, that the initial nuclear ploidy increases which were observed in the liver following DMN administration, did not involve an intermediate binucleate cell system but rather an arrest, or permanent block, in the cell cycle between the end of DNA synthesis and the onset of mitosis. Over the first four days following DMN treatment this hypothesis would, qualitatively at least, explain the formation of the tetraploid nuclei from the diploid nuclear population.

The small rise in octoploid nuclei over this period could have been, in a like fashion, derived from the small numbers of tetraploid nuclei as found in control neonate livers at this time.

The origin of all the 8 and 16N nuclei, which were seen in significant numbers on days fourteen and twenty-one of the experiment is not so easily explained. Since the development of the binucleate cells containing hyperdiploid nuclei would appear to precede, or at least accompany, the formation of the octoploid and 16N nuclear ploidy it would seem reasonable to assume that binucleate cell mitosis could contribute to the formation of higher levels of nuclear ploidy at this stage. The alternative to this suggestion is that the cells damaged by the DMN treatment are blocked a second or possibly a third time in G_2 of the cell cycle as repeated attempts are made by these cells to divide.

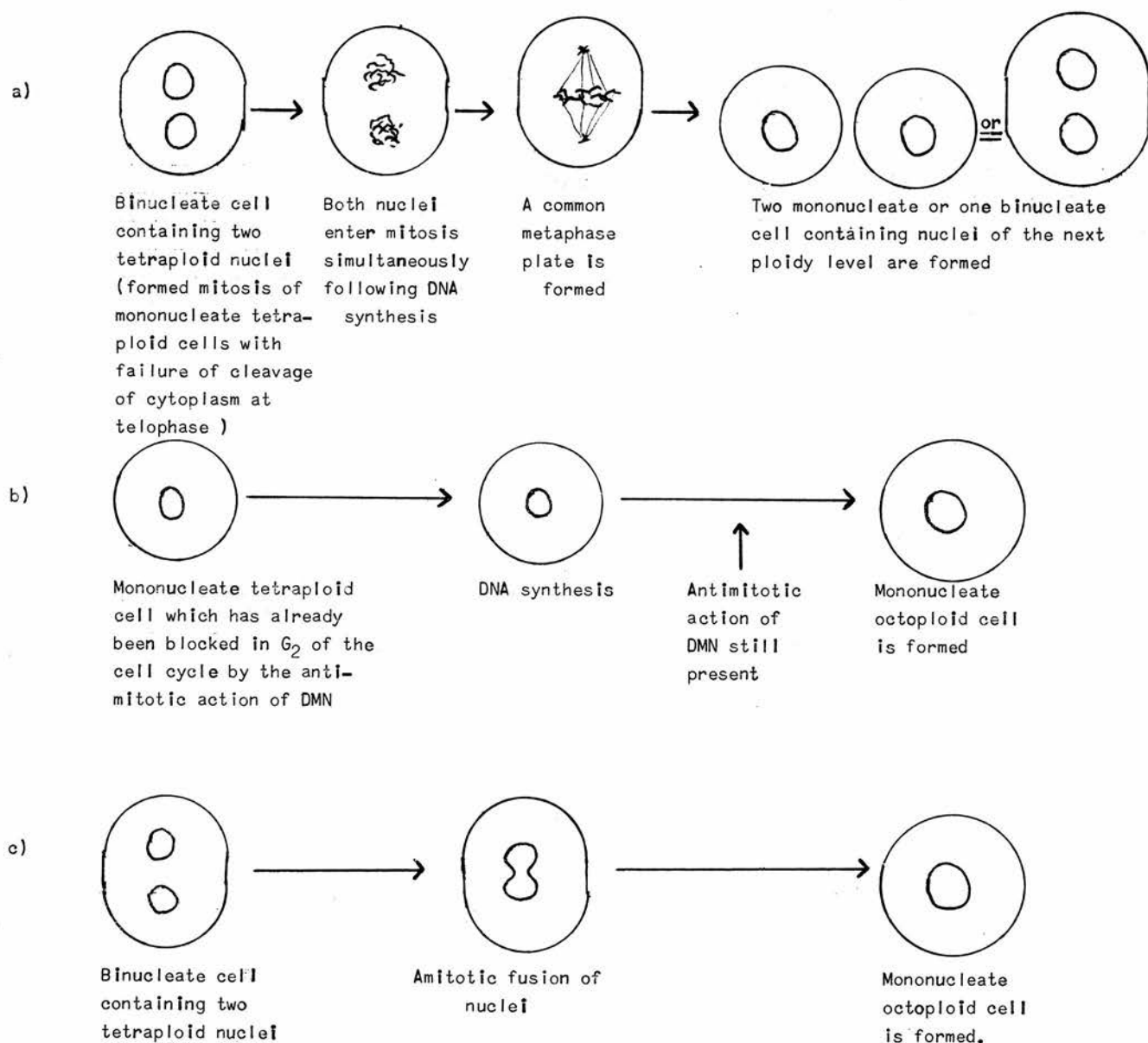
The finding of binucleate cells with two hyperdiploid nuclei can be taken as evidence of the ability of a proportion of the mononucleate hyperdiploid cells induced by the DMN treatment to enter mitosis eventually.

Such evidence of mitosis indicates that some cells which were blocked in G_2 of the cell cycle on their first attempt at division, were subsequently able to reduplicate their DNA content at a later time and on this occasion were able to achieve division of the nucleus but not division of the cytoplasm. In this way a binucleate cell containing two hyperdiploid nuclei would be formed.

Another possible explanation for the increase in nuclear ploidy must be mentioned. The concept of increased nuclear size by nuclear

fusion has been put forward by other workers (Pfühl 1939). The next experiment described in this chapter yielded no evidence to support such a mechanism. Text-Fig. 8.3 illustrates diagrammatically the three possibilities of hypertetraploid nuclear formation observed over the latter half of Experiment 8.1.

Text-Fig. 8.3 The possibilities with regard to the formation of hypertetraploid nuclei from days 14 to 21 of Experiment 8.1. (The evidence presented in this chapter suggests that mechanism a) is the most likely).



With the realisation that the formation of polyploid nuclei in the neonate liver following DMN administration was through a mechanism involving an arrest or block in the cell cycle it was obvious that an investigation of the mitotic activity of the parenchymal cell population should be undertaken. In Experiment 8.1 it was seen that the polyploid nuclei reached thirty-sevenpercent of the total nuclear population and it was hoped that a study of the mitotic activity would indicate more precisely the nature of this large increase.

Experiment 8.3

An investigation of the influence of a single dose of dimethylnitrosamine on the mitotic activity of the parenchymal cells.

Method

Litters of 2-day old rats were divided into two groups so that, as far as possible, the sexes and litters were equally distributed between the groups. At 10 a.m. one group was dosed with 10 mg DMN/kg body weight and the other group with 20 μ l physiological saline by intraperitoneal injection. The animals were administered colcemid at six hours and then at 24 hour intervals up to the seventh day following the initial administration. The animals were killed and the livers processed and appraised for mitotic activity as already described (p 93). This procedure was repeated as the litters became available until there were at least five and in most cases six animals represented in each group at each time interval. Two additional control groups were included at one and two days of age so that the study of mitosis following birth would be more complete.

Results

Table 8.6 sets out the mean and standard deviation of the percentage metaphase mitotic figures scored in the control and DMN treated groups (for individual values observed see Table 5 in Appendix).

Text-Fig. 8.4 shows, in graphical form, the mitotic activity of the two groups.

Throughout the scanning of sections for mitoses no hepatocytes with constricted nuclei were seen; had they existed these nuclei could have been taken as evidence of amitosis with increase in nuclear ploidy by a process involving fusion of nuclei.

Statistical analysis

The means of treated and control groups from age 2-9 days were tested for interaction (Snedecor and Cochran, 1956).

$$F = 6.16 \quad n_1 = 7 \quad n_2 = 79 \quad p < 0.001$$

The highly significant interaction showed that both treated and control groups needed to be further analysed separately.

Further analysis of the control values showed that the greater proportion of the variation between groups could be accounted for in a quadratic form ($p < 0.01$).

Further analysis of the values from the DMN treated animals showed that the greater proportion of variation between groups could be accounted for by linear regression ($p < 0.001$).

Comparison of the treated and control means at each interval showed that only the first three treated groups differed significantly from the corresponding control means ($p < .05$) (See Table 11 Appendix).

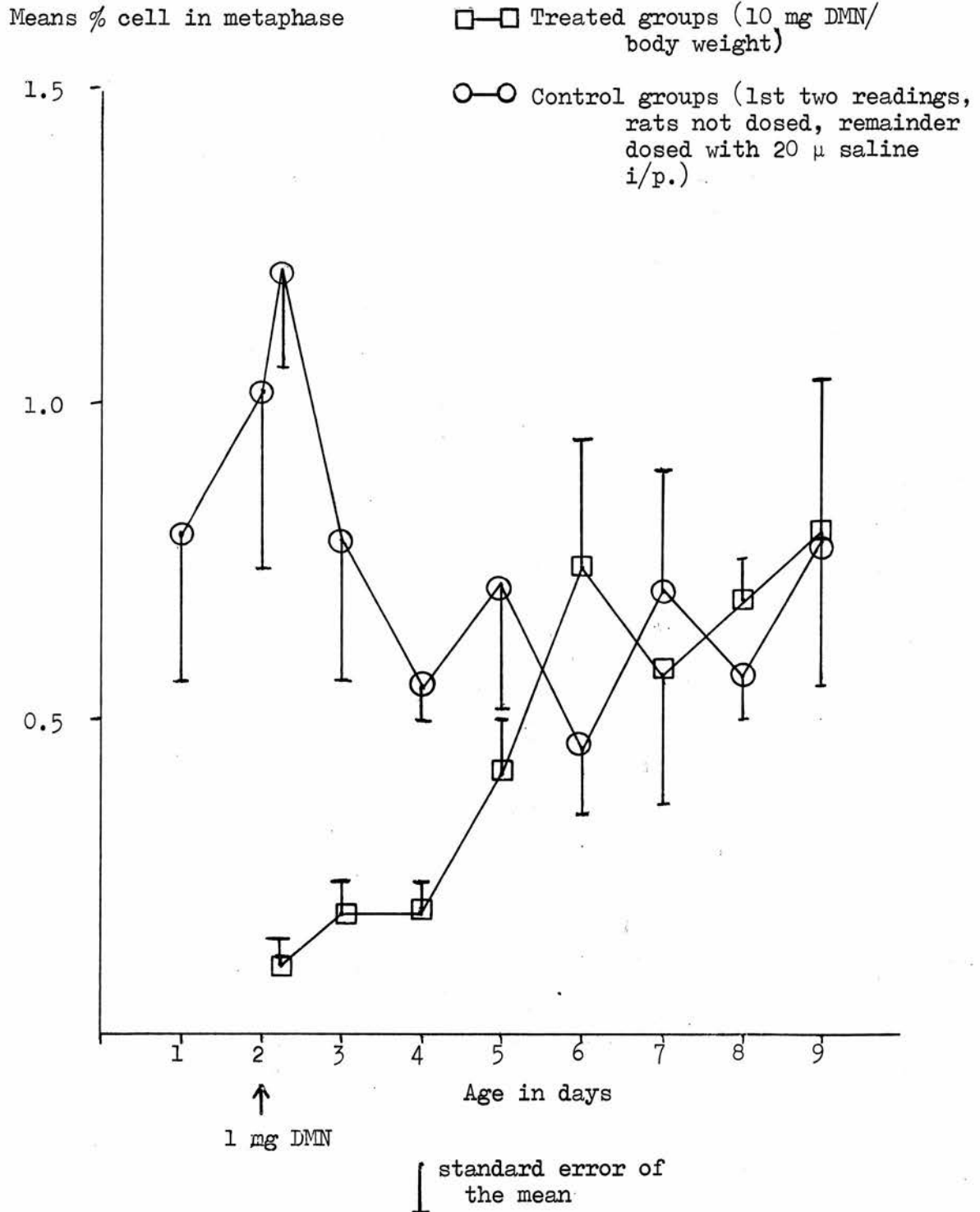
Table 8.6

Mean and standard deviation of nuclei in metaphase expressed as a percentage of all hepatocyte nuclei scored in groups of Ash-Wistar rats dosed at 2 days of age and killed at varying times thereafter.

Age in days	Dosage regimen	
	Control group (20 μ l saline/rat)	Treated group (10 mg DMN/kg body wt.)
1	0.79 \pm 0.50 *	
2	1.01 \pm 0.72 *	
2 $\frac{1}{4}$	1.21 \pm 0.33	0.11 \pm 0.09
3	0.77 \pm 0.48	0.19 \pm 0.13
4	0.55 \pm 0.14	0.19 \pm 0.13
5	0.71 \pm 0.42	0.41 \pm 0.25
6	0.45 \pm 0.24	0.74 \pm 0.48
7	0.70 \pm 0.39	0.57 \pm 0.45
8	0.57 \pm 0.17	0.69 \pm 0.15
9	0.78 \pm 0.58	0.79 \pm 0.59

* These control animals were not dosed with saline
3000 hepatocyte nuclei were scored/rat liver sample.

Text-Fig. 8.4 Percentage means of metaphase mitotic figures observed in control and DMN treated rats injected at 2 days of age and killed at varying times thereafter.



Discussion

The statistical evaluation of the control mitotic figures from day 2 of the experiment indicated that there was greater mitotic activity at two days of age when compared to the mitotic activity at remaining ages studied. After two days of age the mitotic activity remained between approximately 0.5 and 0.7 percent up to the ninth day of age. A more detailed discussion of these results in relation to findings on mitotic activity in normal liver growth by other workers may be found in the next chapter (p 250).

It is apparent that, following DMN administration, there was a profound depression in the mitotic activity of the parenchymal cells, (Text-Fig. 8.4). This depression was most marked for the first two days of the experiment and was apparent as early as six to nine hours following the administration of DMN. From the second to the third day of the experiment the mitotic activity returned to control levels as evaluated by statistical analysis. This aspect is discussed in more detail on page 210 of the chapter, since while the nuclear ploidy increases up to at least day four of the experiment the mitotic inhibition is significantly depressed for two days only.

No evidence of fusion of cells or nuclei was observed and it must therefore be concluded that the increase in nuclear size and DNA content was due to a block or an arrest in the G_2 stage of the cell cycle. This block, however, was not absolute as some hepatocyte mitoses were observed even in the most pronounced stage of mitotic depression produced by DMN. It would seem, therefore, that following the administration of DMN, a proportion of hepatocytes passing through

DNA synthesis failed to enter mitosis.

In the preliminary work with DMN (Chapter 6) it was seen that 10 mg/kg body weight led to necrosis of hepatocytes. Loss of hepatocytes in the adult rat liver through partial hepatectomy (Grisham 1962; Fabrikant 1969) or chemical damage (Melvin 1968) usually evokes a wave of mitosis of the hepatocyte population as a repair process. It is seen from this experiment that this is not so in the case of DMN. To explain the high level of increased nuclear ploidy following DMN damage, however, it would seem reasonable to postulate that the repair processes must have been initiated with a subsequent increase of DNA synthesis in the liver cells. Confirmation of this increase of DNA synthesis was obtained from the next experiment.

The previous experiment showed that the level of mitosis as normally seen in the neonate liver could not, by itself, account for the high level of polyploidy induced by DMN treatment. A study of DNA synthesis in hepatocytes was, therefore, carried out with regard to this problem.

Experiment 8.4

An investigation of the influence of a single dose of dimethylnitrosamine on DNA synthesis in hepatocyte nuclei.

Method

Litters of Ash-Wistar rats were dosed by intraperitoneal injection at 10 a.m. at two days of age with 10 mg DMN/kg body weight or 20 μ l physiological saline. Dosing was arranged as far as possible so that the sexes and litters were equally distributed within the groups. The animals were killed at seven hours, 24 hours and thereafter at 24 hourly intervals until the fifth day following the initial injection. Animals were injected with tritiated thymidine at 1 μ c/gm body weight one hour before sacrifice. This procedure was repeated as the litters became available until there were four animals represented in each of the DMN and control groups. After the animals were killed, the individual liver samples from each animal were processed for autoradiography as described (p 119). Three thousand hepatocyte nuclei were counted for each liver sample and scored either as labelled or as unlabelled with tritium. A nucleus was scored as labelled if there were more than four grains directly overlying it in the emulsion.

Results

Table 8.7 sets out the percentage means of labelled nuclei in the control and treated groups.

Text-Fig. 8.5 illustrates the level of DNA synthetic activity of hepatocytes in control and treated groups (Table 6 in the Appendix shows the results obtained for each animal).

Statistical analysis

i) An analysis of variance on both DMN treated and control groups showed there to be significant differences between means.

$$F = 8.65 \quad n_1 = 11 \quad n_2 = 36 \quad p = < 0.001$$

Discussion

Following a single dose of DMN to the neonate rat, there would appear to be an initial inhibition of DNA synthesis in hepatocytes observed at the seven hour period studied, $p < 0.02$ when the two means at the seven hour time interval were compared by the Student's t test. By twenty-four hours, however, a marked increase in DNA synthesis occurs in the parenchymal cell population which persists for at least a further twenty-four hours. The level of DNA synthesis in this study was seen to have returned to control levels by the third day following injection. Histological evaluation of these livers in the DMN dosed groups revealed necrosis at one and two days of the experiment.

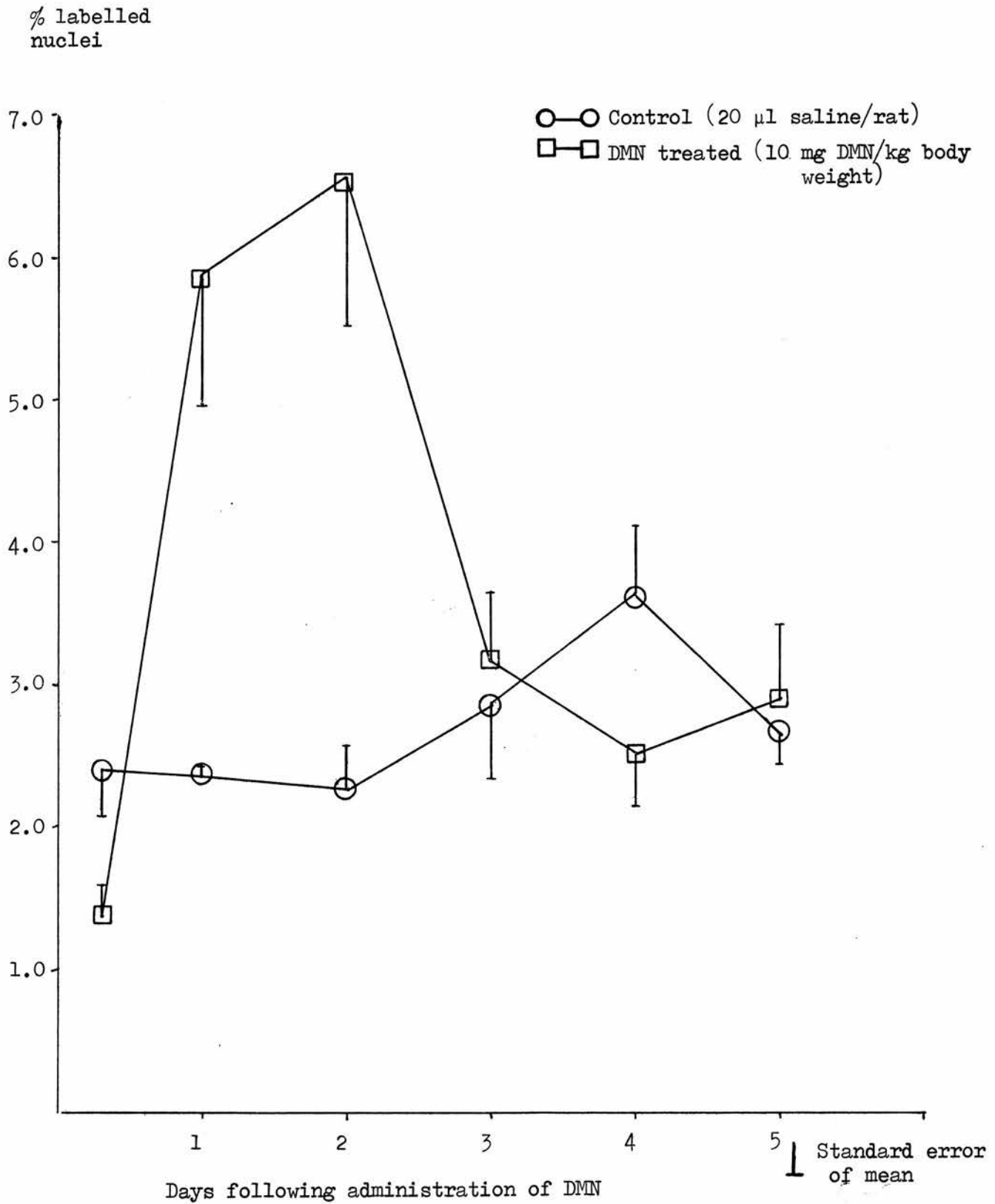
On the basis of this experiment the large increase in polyploid nuclei which was seen after DMN administration is due to a wave of DNA synthesis. Many of the hepatocytes fail to pass through the G_2 stage of the cell cycle into mitosis.

Table 8.7

Percentage means of H³thymidine labelled hepatocyte nuclei in groups of Ash-Wistar rats dosed at two days of age and killed at varying times thereafter

Time between dosing and death	Dosage regimen	
	Control group (20 μ l saline i/p)	Treated group (10 mg DMN/kg body weight)
	% labelled hepatocyte nuclei	
7 hours	2.40 \pm 0.54	1.38 \pm 0.44
1 day	2.37 \pm 0.33	5.98 \pm 1.98
2 days	2.29 \pm 0.60	6.57 \pm 2.02
3 days	2.89 \pm 1.09	3.19 \pm 0.92
4 days	3.63 \pm 1.02	2.52 \pm 0.70
5 days	2.68 \pm 0.47	2.90 \pm 1.02

Text-Fig. 8.5 Graphical representation of the H^3 thymidine labelled hepatocyte nuclei in livers of control and DMN treated animals injected at 2 days of age and killed at varying times thereafter.



The wave of DNA synthesis is undoubtedly associated with an attempt by the liver to repair the damage and cell loss brought about by the DMN treatment the nature of which was described in Chapter 6. These results indicate that repair of the necrotic lesion induced by DMN takes place to a considerable extent by increase in cell size as opposed to restoration of cell number.

General discussion on Chapter 8

The results of the experiments described in this chapter make it clear that DMN treatment initiates a reparative wave of DNA synthesis in the liver, but a large proportion of the hepatocytes are subsequently unable to enter mitosis. An increase in ploidy thus ensues.

The increase in hyperdiploid cells continues until at least the fourth day after treatment. Statistical evaluation of the antimitotic effect, on the other hand, shows that, by day three, the mitotic rate of the hepatocytes has returned to control levels. Thus, the increase in nuclear ploidy from day three to day four, as seen in Experiment 6.1, has not been explained. It was seen in Experiment 8.3, however, that mitotic activity in the neonate liver of the rat can vary considerably from one individual animal to another. It is conceivable, therefore, that had larger numbers of rats been used for treated and control groups, a significant depression of mitotic activity might have been established on day three of the experiment. An experiment described in a later chapter (p 281) did in fact indicate very strongly that the antimitotic effect of DMN persists for up to four days following a single administration to the neonate rat.

The following discussion compares the nuclear enlargement induced by DMN with that induced by other hepatotoxins which would also appear to act through an antimitotic mechanism. In the light of findings by other workers, a mechanism of action involving specific damage to DNA is put forward as an explanation of the arrest of the hepatocyte in G₂ of the cell cycle. The duration of the antimitotic effect in

neonate rat liver following a single administration of DMN is then considered in more detail. Finally the fate of those cells blocked in G_2 of the cell cycle is discussed and brief reference made to repair of the damage brought about by DMN in relation to its antimitotic action.

i) Nuclear enlargement brought about by DMN and other hepatotoxins

Nuclear enlargement in the parenchymal cell population of the liver as a result of DMN administration was observed in histological section (Barnes and Magee, 1954). Using microspectrophotometry Christie and LePage (1961) showed that the enlargement was associated with a concomitant increase in nuclear DNA content. An explanation of the mechanism by which DMN brings about a shift in nuclear ploidy was not given by the above authors.

A scrutiny of the literature indicates that dimethylnitrosamine is by no means the only hepatotoxin which is capable of inhibiting mitosis. Pyrrolizidine alkaloids bring about a striking enlargement of hepatocytes and their nuclei (Selzer et al., 1951; Bull, 1955; Schoental and Magee, 1957; Bull and Dick, 1959; Peterson, 1965). Jago (1969) demonstrated that lasiocarpine produced this effect by inhibiting mitosis. If given at necrotoxic doses so as to initiate a wave of DNA synthesis then accumulation of megalocytosis was rapid; alternatively, if given at below a necrotoxic level the onset was more insidious.

Dimethylaminoazobenzene also causes the formation of enlarged hepatocytes and nuclei in the liver of the rat (Orr, 1948). Evidence

has since been presented that this enlargement is due to an antimitotic effect induced in the hepatocyte population. In this connection it has been shown that when partial hepatectomy is performed during prolonged administration of DAB there is an inhibition of the expected restorative mitotic wave as compared to partially hepatectomised control rats (Brody, 1960; Banerjee, 1965). Similar findings were reported in rats fed the closely related azo compound, 3'-methyl-4-dimethyl-aminoazobenzene (Stitch and Maini, 1962). Furthermore, Simard et al. (1968) were able to show that during prolonged administration of DAB in the diet of rats, a proportion of the hepatocytes exhibited a greatly protracted G₂ phase of the cell cycle which led to increased nuclear ploidy. These latter findings are in agreement with an antimitotic effect where cells reduplicate their DNA content but do not proceed further through that particular cell cycle.

Yet another compound which has been found to produce enlarged hepatocyte nuclei is aflatoxin (Butler, 1964). It would appear that this hepatotoxin brings about such nuclear enlargement by a similar mechanism as the above mentioned compounds. Following a single administration of aflatoxin an inhibition of the mitotic activity of the parenchymal cell population has been reported (Rogers and Newberne, 1967) which persists for two to three days. This work also demonstrated an inhibition of hepatocyte DNA synthesis which persisted for two-three days following a single injection. This is in marked contrast to the effect of DMN on this parameter found in the work of this thesis.

ii) Mechanism of action

In view of the similarities found in the effects of these compounds on hepatocytes it is tempting to look for common mechanisms of action through which the normal cell cycle is disrupted in each case.

Direct attack on the nucleus with the initial lesion being the alkylation of DNA, has recently been suggested as the mechanism by which lasiocarpine, a pyrrolizidine alkaloid, induces nuclear enlargement (Jago, 1969; McLean, 1970).

In this connection, all four compounds mentioned above have been shown to induce ultrastructural changes in the nucleus. The most obvious aberrations are produced within the nucleolus, with DMN and DAB inducing a change termed microsegregation and lasiocarpine and aflatoxin macrosegregation. The effects represent essentially a rearrangement of the fibrillar components of the nucleolus but their significance is not apparent as yet (Svoboda and Higginson, 1966; Svoboda and Reddy, 1969).

Biochemical data on the interaction of these compounds and their metabolites with DNA yields more information however. DMN, in addition to alkylating protein and RNA, was shown in vivo to methylate the guanidine moiety of DNA (Magee and Farber, 1962). Similarly, diethyl-nitrosamine which also induces nuclear enlargement of hepatocytes (Hobik and Grundman, 1962), ethylates the guanidine moiety of RNA in the liver (Magee and Lee, 1963). Although alkylation of DNA itself has not been demonstrated in the newborn rat following DMN administration, methylation of RNA has been shown to increase over the first three days

of life (Lee and Spencer, 1964). Thus, if alkylation of RNA occurs at this age it would seem reasonable to argue that methylation of DNA could also take place in the neonate liver. Finally, the mutagenic potential of DMN is thought to be due to its alkylating action on DNA, indicating that profound genetic disturbances can be induced by this kind of interaction (Pasternak, 1962; Geissler, 1962).

One of the pyrrolizidine alkaloids, heliotrine has been shown to alkylate cysteine in vitro (Culvenor et al., 1962). More recently following administration of radioactively labelled lasiocarpine, DNA has been isolated from the liver with small amounts of this radio activity combined with it (Culvenor et al., 1969). This latter finding was viewed with some caution however as the associated activity was in trace amounts only and thus was not necessarily specific. This author suggested that the toxic activity of this compound was due to the formation of pyrroles and it is now known that pyrroles can act as bifunctional alkylating agents (Mattocks, 1969).

Warwick (1967) and Warwick and Roberts (1967) have demonstrated that there is persistent binding of radioactive metabolites of DAB to DNA in vivo. This finding involved the administration of the tritium labelled compound and was not attributed to any spurious incorporation of small metabolites such as tritiated water into the natural bases during synthesis. Such a persistent interaction was suggested by the above workers to result in a high level of mitotic abnormalities which previous workers (Maini and Stitch, 1961) had recorded as long as six months after the cessation of the feeding of a diet containing DAB.

Finally, the binding of aflatoxin to DNA has been observed both in vitro (Clifford and Rees, 1966; Sporn et al., 1966) and in vivo (Lijinsky, 1968).

The above work, which could be taken as evidence that chemical damage of DNA may result in adverse repercussions on the control of the normal division of the cell, is further supported by observations on radiation effects. That radiation damage may evoke chromosomal defects is indicated by the large number of mitotic abnormalities in regenerating rat liver following x-irradiation (Albert, 1958; Curtis et al., 1964; Leong et al., 1961). This damage to hereditary material is thought to lead to the mutations induced by x-irradiation in rats (Brown, 1964; Newcombe and McGregor, 1965) and by β -rays emitted by tritiated thymidine in Drosophila (Stromness and Kvellard, 1963). It is relevant that injection of relatively high levels of tritiated thymidine can lead to increased nuclear polyploidy in the liver of the neonate rat (Post and Hoffman, 1967) and that x-irradiation of the whole animal or organ leads to an inhibition of mitosis with increased nuclear polyploidy after partial hepatectomy (Leong et al., 1961; Webber and Stitch, 1965; Williams et al., 1952; Fabrikant, 1967; 1969). At least two publications have contained postulates that the increased ploidy following irradiation in vivo (Post and Hoffman, 1967) and in HeLa cells in vitro (Puck and Marcus, 1956) is due to direct chromosomal damage.

The hypothesis stated above, which suggests that damage to DNA molecules in cells can adversely effect cell division, is supported by these results obtained with hepatotoxins, and finds further support

from observations on irradiated bacterial cells. Doses of radiation which are too small to kill cells rapidly may have a marked effect on the ability of those cells to divide. When a strain of Escherichia coli was irradiated with γ -rays, cell division ceased and the total number of organisms remained constant; meanwhile the cells increased in length so that growth rates in control and irradiated cultures were similar (Lea, 1955).

Returning to mammalian systems the effect of the alkylating agent, Myleran (Burroughs Wellcome and Co. 1.4, dimethanesulfonylbutane) supports the above concept since its action on the lens epithelium of the eye is one of inhibition of mitosis in the rapidly proliferating equatorial zone, without in any way affecting DNA synthesis and in consequence producing polyploid nuclei. (Grimes and Sallmann, 1966).

These observations could, therefore, be taken to support the view that the onset of mitosis is dependent to some degree on the integrity of the DNA molecule and that the inhibition seen with DMN is mediated in some way through the damage brought about by alkylation of DNA or by incomplete repair of such damage as discussed below. It should be emphasised however that interaction of hepatotoxic substances with DNA has attracted much attention over the past decade in an attempt to explain their neoplasia and mutation inducing potential and to some extent other cellular interactions have been relatively ignored. There is no valid reason to suppose, at the present time, that damage to other cell constituents could not be the initial lesions underlying such effects. However further discussion is best left until the concluding discussion of this thesis, one section of which outlines the

possible association of inhibition of protein synthesis with nuclear enlargement.

iii) The fate of hepatocytes blocked in G₂ of the cell cycle

The fate of at least most of the cells which were blocked in the G₂ stage of the cell cycle is apparent when the results of the experiments carried out so far are considered in more detail.

It has already been mentioned (p 197) that the formation of binucleate cells with two hyperdiploid nuclei is an indication that arrested cells are able to pass through nuclear division at a later stage. Further evidence in support of this concept comes from a consideration of the growth rate of the liver. Following administration of DMN, experiments have shown that the weight of this organ increases in absolute terms by approximately 800 percent over a period of 21 days. On this basis, if all or a large proportion of the mononucleate hyperdiploid cells formed during the first four days following DMN treatment survived, but did not divide further, they would have been grossly diluted out over the ensuing three weeks by division of the diploid population of hepatocytes. No such gross dilution was observed and therefore although many cells were prevented from entering mitosis on their first attempt most at least were capable of division at a later stage.

A close scrutiny of Text-Fig. 8.1, however, reveals that the relative proportion of hyperdiploid nuclei did in fact decrease from day four of the experiment when they constituted 37.25% of the nuclear population to a level of 26.4% by day twenty-one. There are four

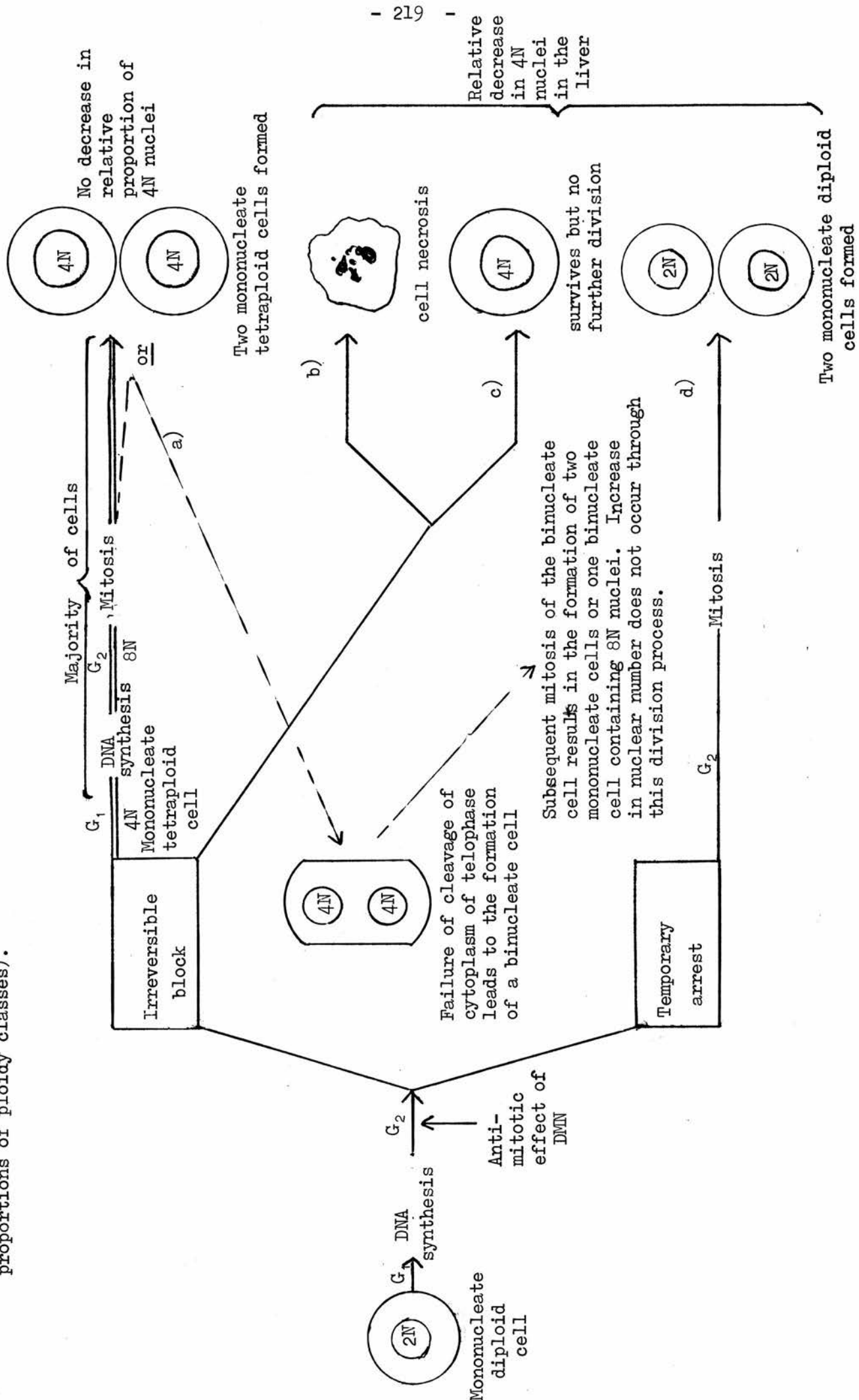
possible explanations of this decrease. These explanations are based on the premise that if the different nuclear ploidy populations seen by day four of the experiment did not show differential replicating patterns then the relative proportions of ploidy classes would remain constant up to day twenty-one. (The control ploidy figures of Experiment 8.1 indicate that tetraploid nuclei formed through normal physiological processes would not have contributed to the DMN induced hyperdiploid nuclei over this period). The four possible explanations of the dilution are:

- a) It was suggested in the discussion of Experiment 8.2 (p 196) that the increase in 8N and 16N nuclei seen over the latter half of the experiment could have been due, in part, to the formation of binucleate cells containing two hyperdiploid nuclei (see Text-Fig. 8.3a). Subsequent DNA synthesis and mitosis of these binucleate cells leads to increase in cell and nuclear size but not to an increase in nuclear number and the relative proportion of hyperdiploid nuclei would thus fall.
- b) A proportion of the hepatocytes blocked in G_2 by the DMN treatment eventually undergo necrosis and are lost from the hyperdiploid population.
- c) Some of the hyperdiploid cells remain permanently excluded from the normal hepatocyte replicating pattern resulting in some dilution of their numbers.
- d) A proportion of the hyperdiploid cells formed by the action of DMN were in fact in a transient but prolonged G_2 stage of the cell cycle. They eventually pass into mitosis to give two daughter nuclei, the ploidy of which returns to the level of the parent nucleus prior to DMN treatment.

All five possible fates of the blocked cells is represented diagrammatically in Text-Fig. 8.6.

Text-Fig. 8.6 Diagrammatic representation of possible fates of mononucleate tetraploid cells induced in the liver by DMN treatment.

(When consulting this diagram it should be remembered that the remainder of the hepatocyte population unaffected by DMN treatment are assumed to be able to divide normally which affects the relative proportions of ploidy classes).



It would seem justifiable to argue that the formation of binucleate cells and their subsequent division could have led to the increased numbers of 8N and 16N nuclei seen in the latter half of Experiment 8.1 but at the same time resulted in a relative decrease in the hyperdiploid nuclear population. However and experiment described in Chapter 12 suggested that mechanisms c) or d) could also have contributed to this dilution.

The argument above which lays stress on the fact that most of the hyperdiploid cells formed by DMN treatment were capable of division at a later stage raises the question of whether there is a temporary or permanent arrest of the cell cycle in the G_2 stage. Simard et al. (1968) termed the effect of mitotic inhibition of hepatocytes seen during DAB administration as one of arrest in G_2 of the cell cycle. As this work gave no indication of the subsequent fate of these cells following such an 'arrest' this would appear to be valid terminology. However, the use of the word arrest would seem to imply that the polyploid nuclei are in a prolonged but transient G_2 phase in the cell cycle and that they have the capability of eventually entering mitosis. In this way two daughter nuclei with ploidy values equal to the parent nucleus prior to DNA synthesis would be formed. Such G_2 populations have been recognised in other tissues such as the epithelial cells of the skin of the mouse (Gelfant, 1962) where they were taken to represent a reserve of cells capable of immediate further division following local injury. The effect produced by DMN on the majority of the parenchymal cells of the neonate rat liver is not of this kind however. Following the arrest of the cell in G_2 , normal intervening mitosis does not occur and the cells pass into a further S phase and only then in the majority of cases pass through nuclear division. Thus the

initial increase in ploidy induced by DMN is in the majority of cells, a genuine irreversible shift rather than one of a transient nature.

It has also been argued above, that the inhibitive effect of a single dose of DMN is on one mitotic cycle only. This is in marked contrast to the effect produced by the hepatotoxic pyrrolizidine alkaloids where, for example, a single dose of lasiocarpine leads to continued megalocytosis over many months (Schoental and Magee, 1957; Nolan et al., 1966). Although the role of the binucleate cell has not been explored in this prolonged action, the persistent antimitotic effect (Downing and Peterson, 1968; Jago, 1969; Culvenor et al., 1969) following single administration indicates an inhibitive effect on repeated attempts of the cell to divide. This is not to imply that division of pyrrolizidine induced megalocytes is impossible since these cells have been observed in mitosis (Schoental and Magee, 1959) although they often had an abnormal appearance in histological section.

iv) Duration of effect of DMN in neonate liver

Experiments in this chapter have shown that the effect of DMN on the cell cycle, as evaluated by the rate of development of polyploid nuclei, persisted for up to four days following administration of the compound. There would appear to be two possibilities regarding the duration of this effect.

a) The duration of effect could reflect the persistence of DMN in the liver of the neonate with relatively slow metabolism producing a mitotic toxic principle over this period of time. This would seem to be most unlikely in view of the finding of Terracini and Magee (1964) that, following administration of DMN to rats less than twenty-

four hours old, no significant trace of the unchanged molecule could be found in the tissues twenty-four hours later. Evidence of metabolism of DMN at this age was demonstrated in this work by the expiration of (^{14}C) carbon dioxide following injection of (^{14}C) labelled dimethylnitrosamine.

b) The second possibility and the one which would seem most likely is that, following administration of dimethylnitrosamine, a large proportion of the hepatocytes are more or less equally susceptible and damaged to the same extent by the mitotic inhibitive metabolite. Some of these cells which are in late G_1 of the cell cycle, or within a few hours are involved in the reparative wave of DNA synthesis, are unable to repair the damage caused by DMN in time and so do not pass through normal mitosis, being blocked in the G_2 phase. As the time from the administration of DMN increases, cells are able to effect the necessary repair to enable them to enter mitosis and the rate of accumulation of polyploid nuclei decreases. By day four of the experiment most cells are passing through a normal division cycle. In a similar fashion, those cells which are actually blocked in G_2 and become polyploid, eventually repair the damage induced by DMN and are then able to enter mitosis following a second reduplication of their DNA content. This hypothesis, which satisfactorily explains the decreasing rate of accumulation of polyploid nuclei and the ability of DMN induced polyploid nuclei to pass through further mitoses is discussed further in the concluding chapter of this thesis.

The conclusions which may be drawn from the experimental work in

this chapter are as follows:-

In the neonate rat a single necrotoxic dose of dimethylnitrosamine induces a wave of DNA synthesis in the hepatocyte population; the majority of these cells are blocked in the G₂ stage of the cell cycle and a shift in nuclear ploidy is observed. With single doses DMN inhibits the first attempt at mitosis but the majority of subsequent divisions appear to be carried through normally.

A mechanisms of action involving damage to DNA has been postulated in the light of findings by other experimentalists on enlargement of nuclei following other hepatotoxic procedures. The duration of the antimitotic effect of DMN following a single injection has been suggested to be due to the relative ability of hepatocytes to repair such damage prior to mitosis.

The fate of a small proportion of hyperdiploid nuclei formed from the DMN mitotic inhibition remains in doubt.

CHAPTER 9

THE NATURE OF NUCLEAR ENLARGEMENT FOLLOWING THE
ADMINISTRATION OF CARBON TETRACHLORIDE

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The experiments described in Chapter 6 established that the nuclear enlargement seen in the liver following DMN administration was brought about through a mechanism involving a block in G_2 of the cell cycle. The probability that other hepatotoxic agents might act in a similar fashion was discussed. Furthermore, it was indicated that interaction with DNA could be a significant feature in such an interference with the cell cycle.

There are other chemicals, such as carbon tetrachloride and thiocetamide, which have not been shown to interact with DNA and yet bring about nuclear enlargement in the liver. In both this chapter and the next it will be shown that the mechanism by which these compounds bring about this effect is entirely different from that demonstrated with DMN and involves an intermediate binucleate cell system.

Experiments were designed to investigate the mitotic activity, changes in the proportion of binucleate and mononucleate cells and changes in nuclear ploidy in the hepatocytes of 4-day old and 28-day old rats dosed with carbon tetrachloride.

1. Studies on 4-day old rats

Experiment 9.1

An investigation of the mitotic activity of the hepatocyte population following a single dose of carbon tetrachloride.

Method

Litters of 4-day old rats were divided into two groups so that as nearly as possible the sexes and litters were equally distributed. At 10 a.m. on day 0 of the experiment one group was dosed with 0.1 ml

carbon tetrachloride/kg body weight, and the other group with 20 μ l physiological saline/rat by intraperitoneal injection.

The rats were killed at varying times after this initial injection of CCl_4 and three hours after the injection of colcemid. The livers of each animal were processed and appraised for the mitotic activity of the hepatocytes as described (p 91).

Results

In Table 9.1 are set out the mean percentage metaphase mitotic figures observed in the carbon tetrachloride dosed and control groups respectively. This is illustrated graphically in Text Fig. 9.1a (see Table 7 in the Appendix for individual observations).

Statistical analysis:

An analysis of variance carried out on the control and treated groups revealed a significant difference between means.*

$$F = 38.4 \quad n_1 = 9 \quad n_2 = 50 \quad p < 0.001$$

Histological description:

The histological appearance of the livers was essentially as reported in the preliminary experiments with carbon tetrachloride in rats at this age. The fifteen hour carbon tetrachloride dosed group showed livers in which ballooned cell formation was well advanced but little necrosis was observed at this time. Both features of this hepatocyte change had increased by twenty-four hours and healing of the lesion was well advanced by day two. Restoration of the liver parenchymal cells was complete by the third day following the administration of carbon tetrachloride.

* In the experiments of this chapter the analyses of variance were carried out with both treated and control groups compared within the same analysis.

Table 9.1

Mean and standard deviation of nuclei in meta-
phase expressed as a percentage of all nuclei
counted in groups of Ash-Wistar rats dosed at
4 days of age and killed at varying times
thereafter.

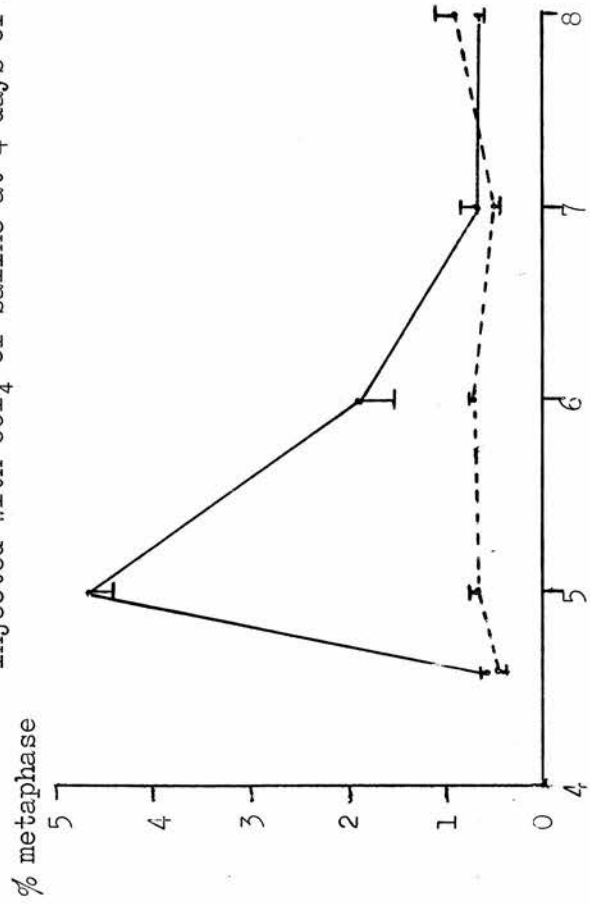
Time between CCl ₄ or NaCl administration and injection of colcemid (Animals killed 3 hrs. after colcemid inj.)	Dosage regimen	
	Control group (20 μl saline i/p)	Treated group (0.1 ml CCl ₄ /kg body weight)
12 hours	0.42 ± 0.11	0.53 ± 0.17
1 day	0.61 ± 0.18	4.62 ± 0.73
2 days	0.70 ± 0.27	1.89 ± 0.94
3 days	0.49 ± 0.16	0.63 ± 0.39
4 days	0.88 ± 0.52	0.62 ± 0.14

3000 hepatocyte nuclei scored/liver sample.

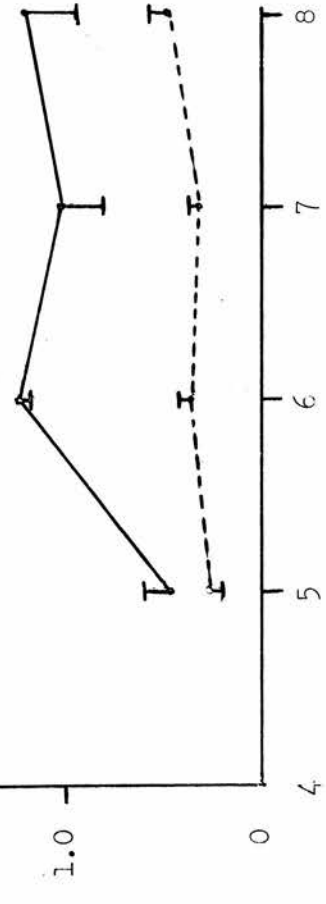
6 rats in each group.

Text-Fig. 9.1

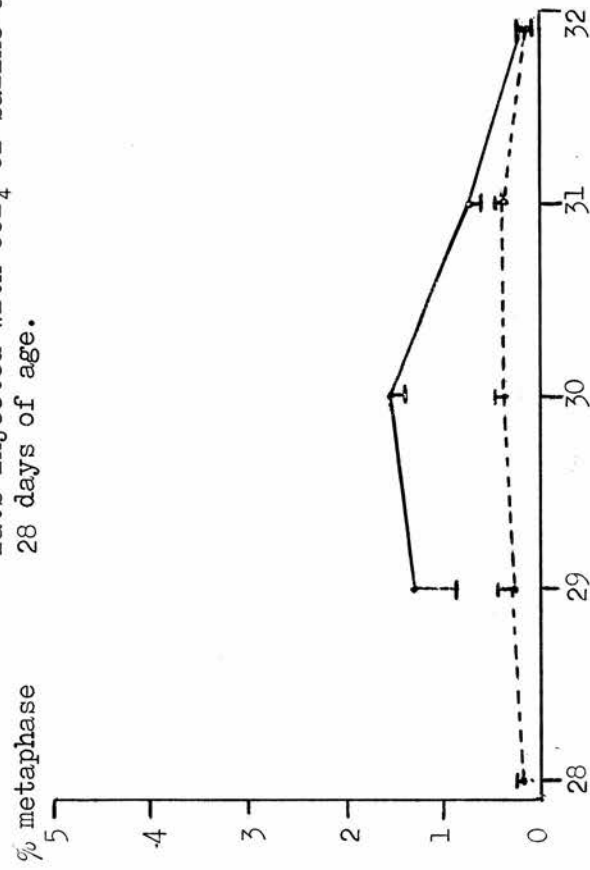
a) Mitotic activity of hepatocytes in rats injected with CCl_4 or saline at 4 days of age.



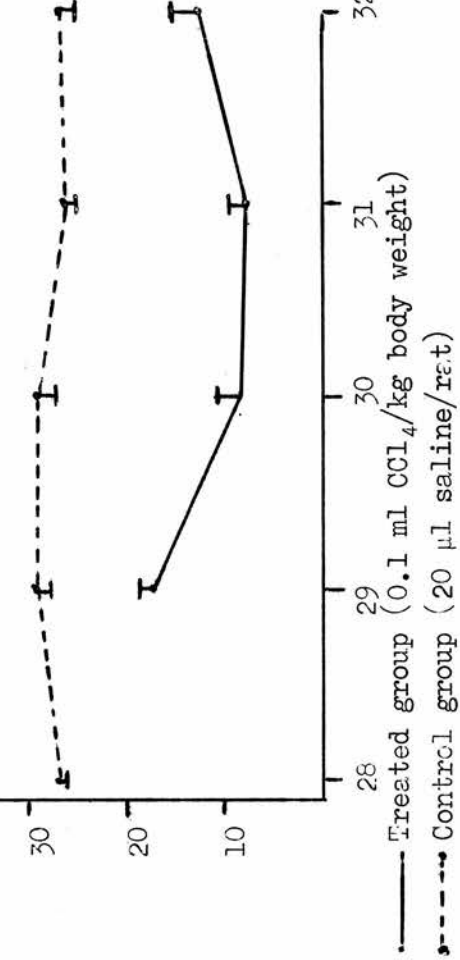
c) Percentage of binucleate hepatocytes in rats injected with CCl_4 or saline at 4 days of age.



b) Mitotic activity of hepatocytes in rats injected with CCl_4 or saline at 28 days of age.



d) Percentage of binucleate hepatocytes in rats injected with CCl_4 or saline at 28 days of age.



Standard error of mean

— Treated group (0.1 ml CCl_4 /kg body weight)
 - - - Control group (20 μ l saline/rat)

Discussion

It may be seen from Text-Fig. 9.1a that there is no significant difference between the means of the control groups at each of the time intervals and that the values obtained are in accordance with those found for control liver mitotic activity in the experiments with dimethylnitrosamine (p 199). There is, however, a marked difference between the means of the one- and two-day treated groups and the means of the remainder of the treated and control groups. It is clear that the administration of carbon tetrachloride to the four-day old rat is followed by a marked increase in the mitotic activity of the parenchymal cells by twenty-four hours. By the second day, this activity declines considerably and returns to control levels by day three of the experiment.

The microscopic appearance of the development and subsequent healing of the lesions induced by the carbon tetrachloride poisoning correlated well with the mitotic activity of the parenchymal cell population. In the absence of a lobular pattern in the four-day old rat liver, it was by no means easy to evaluate the spatial arrangement of mitoses in the regenerative response. My opinion is, that dividing cells were distributed randomly in the periportal and midzonal areas, which were unaffected by the necrotoxic action of carbon tetrachloride.

Experiment 9.2

An investigation of the proportions of mononucleate and binucleate hepatocytes following a single necrotoxic dose of carbon tetrachloride

Method

Litters of rats, four days old, were divided into two groups and dosed as described in Experiment 9.1. The animals were killed at daily intervals until the fourth day following the initial injection so that there were two male and two female rats in the carbon tetrachloride and control groups respectively at each time interval.

The livers from each of the animals were disaggregated as described on p 98 and mononucleate and binucleate cell counts made with the aid of a haemocytometer. A total of two thousand hepatocytes were scored for each liver sample.

Results

In Table 9.2 are set out the mean percentage of binucleate hepatocytes at each time interval for the carbon tetrachloride dosed and control groups respectively. These results may be seen graphically in Text-Fig. 9.1c (see Table 8 in Appendix for individual observations). No binucleate cells containing hyperdiploid nuclei were observed.

Statistical analysis:

An analysis of variance on both treated and control groups revealed a significant difference between the means.

$$F = 11.80 \quad n_1 = 7 \quad n_2 = 24 \quad p < 0.001$$

Table 9.2

Percentage means of mononucleate and binucleate hepatocytes (\pm standard deviation) in groups of Ash-Wistar rats dosed at 4 days of age and killed at varying times thereafter.

Time between dosing and death (in days)	Dosage regimen			
	Control group: 20 μ l saline i/p		Treated group: 0.1 ml CCl ₄ /kg body weight	
	Mononucleate cells	Binucleate cells	Mononucleate cells	Binucleate cells
1	99.77	0.28 \pm 0.10	99.55	0.45 \pm 0.31
2	99.62	0.38 \pm 0.13	98.77	1.23 \pm 0.09
3	99.67	0.33 \pm 0.13	98.99	1.01 \pm 0.48
4	99.52	0.49 \pm 0.18	98.80	1.20 \pm 0.51

2000 hepatocytes scored/liver sample.

Discussion

Experiment 9.2 thus established that two days after a necrotising dose of carbon tetrachloride there was a slight, but nevertheless significant, rise in the relative size of the binucleate cell population of the liver as compared with the proportion seen in control livers. This elevation was maintained over the following forty-eight hours.

These observations indicate that, during an induced wave of mitosis in the hepatocyte population, a small proportion of cells which take part in this mitotic activity effect division of the nucleus, but become binucleate because of non-cleavage of the cytoplasm.

There are two alternatives to this suggestion. The increase in the binucleate population could have been due to fusion of two mononucleate cells, as postulated by Wilson and Leduc (1948), or alternatively, through an amitotic method of equal division by splitting of the nucleus (Clara, 1931; Wilson and Leduc, 1948; Suppon, 1966). No evidence in support of either of these mechanisms was seen in the histological sections of Experiment 9.1.

Experiment 9.3

An investigation into changes in the relative proportions of ploidy classes of hepatocyte nuclei in rats dosed with carbon tetrachloride at four days of age.

Method

A litter of 11 rats, containing suitable numbers of both sexes, was culled shortly after birth so that it consisted of four males and four females (lowest body weights discarded). At four days of age, two males and two females were injected intraperitoneally with 0.1 ml carbon tetrachloride/kg body weight and the remaining two males and two females with 20 μ l physiological saline. Four days after these initial injections the animals were killed, the livers disaggregated and smears prepared and stained with Feulgen aq-light green as described on page 102. The DNA content of one hundred separate nuclei was measured in each liver sample.

Results

In Table 9.3 is shown how the samples of nuclei were distributed between the two ploidy classes in the dosed and control groups. The actual distribution of nuclei according to their DNA content is shown in the histograms of Text-Fig. 9.2

Statistical analysis:

A Student 't' test performed on the data given in Table 9.3 showed that no significance should be attached to the slight difference between the percentage of tetraploid nuclei found in the dosed and control groups respectively.

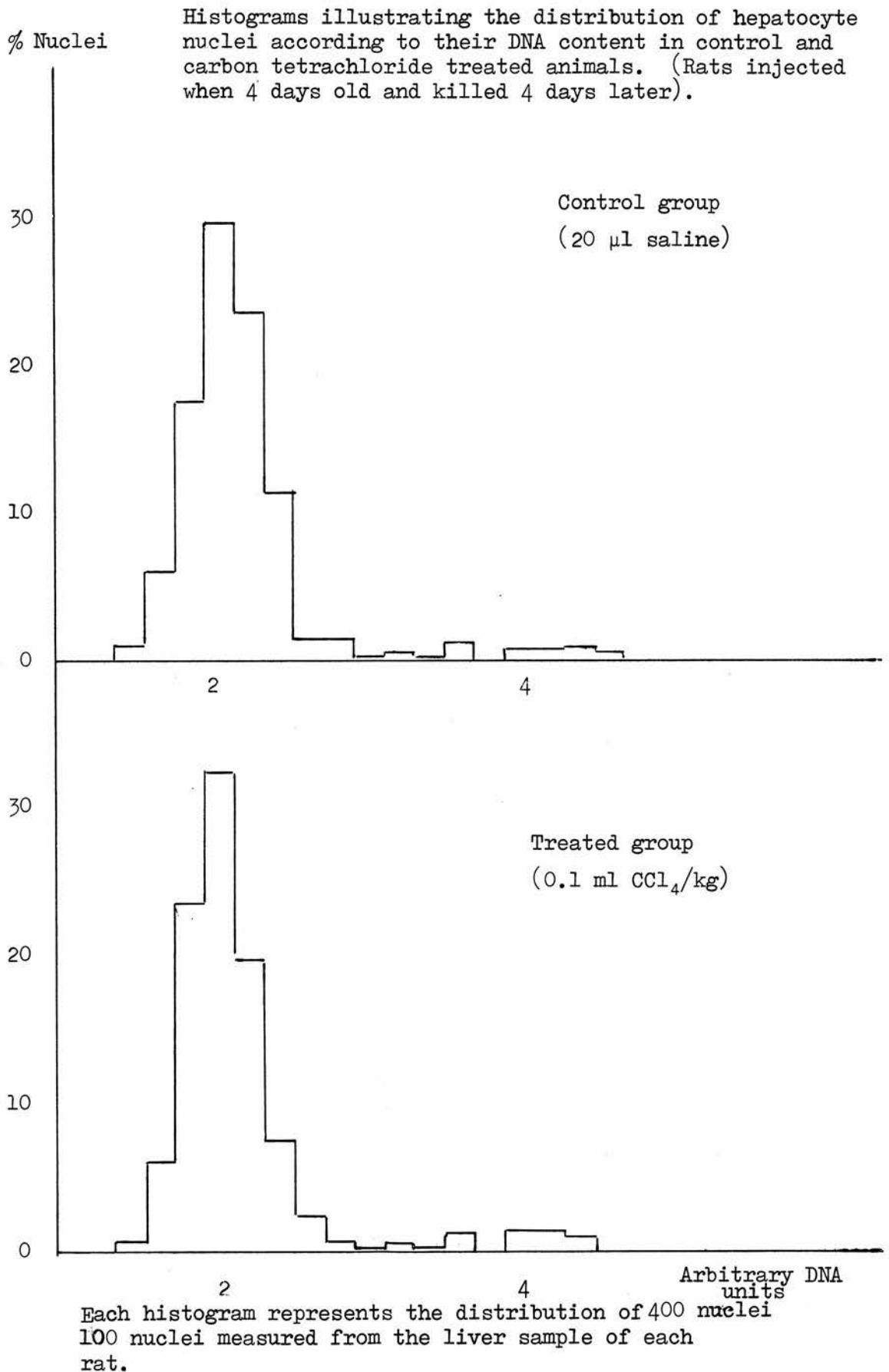
Table 9.3

Means of proportions of nuclear ploidy classes
found in control and carbon tetrachloride groups
of rats dosed at four days of age and killed four
days later.

Dosage regimen	Rat No.	% Diploid nuclei	% Tetraploid nuclei
Control group (20 μ l saline ip)	1	97	3
	2	93	7
	3	96	4
	4	94	6
Means		95	5
Treated group (0.1 ml CCl ₄ / kg body wt.)	1	93	7
	2	95	5
	3	94	6
	4	95	5
Means		94	6

100 nuclei examined for DNA content from each liver sample.

Text-Fig. 9.2



Discussion

From the experiments reported in this chapter the following two facts have emerged. Carbon tetrachloride given to the four-day old rat brought about a regenerative wave of mitosis with a rise in the number of binucleate hepatocytes. There was however no rise in nuclear ploidy over the period studied.

Following partial hepatectomy in the eight to ten day old rat, a procedure which also gives rise to a regenerative wave of mitosis, Nadal and Zajdela (1966), were likewise unable to detect any rise in nuclear ploidy four days post-operatively. However these workers also failed to detect any rise in the binucleate population following partial hepatectomy at this age. This is in contrast to the results of Experiment 9.2 which showed a small but nevertheless significant rise in this cellular population of the neonate liver. This discrepancy might be attributable to the low number of neonatal rats which survived the operative procedures in the work of Nadal and Zajdela (one animal only at eight and ten days of age respectively). On the basis of such low survival it is difficult to draw valid comparisons.

However, the rise in the numbers of binucleate cells following carbon tetrachloride dosing seen in Experiment 9.2 is by no means unexpected when considered in the light of current knowledge on the spatial arrangement of binucleate cells in the liver lobule. McKellar (1949) and Geller (1965) have shown that the distribution of binucleate cells throughout the lobule is by no means random. Specifically McKellar counted more binucleate cells in the intermediate third of the

lobule in rats 14, 23 and 148 days of age and this is in agreement with Geller who also found the majority of binucleate cells in this zone in the 30 gm rat. It would thus seem that binucleate cells are formed predominantly in this area, at least in early life. It is this zonal distribution of binucleate cells coupled with the zonal necrosis of carbon tetrachloride which enables the rise in binucleate cells seen in Experiment 9.2 to be explained. In this connection it has been seen that carbon tetrachloride brings about a centrilobular necrosis in the liver. Thus a loss of centrilobular cells, which are mainly mononucleate, will result in a corresponding relative rise in the proportion of binucleate cells scored in a counting system involving liver disaggregation as used in Experiment 9.2. Since the necrosis due to carbon tetrachloride persists for two days this would explain the rise seen over the first two days of the experiment.

In order to explain the maintenance of the binucleate cell rise over days three and four of the experiment one must turn to evidence dealing with the spatial arrangement of mitoses involved in the regeneration response to carbon tetrachloride. In this connection, observations in the adult mouse following carbon tetrachloride poisoning have shown that DNA synthesis and mitosis occur predominantly in the periportal and midzonal regions of the lobule (Melvin, 1968). Thus, regeneration after carbon tetrachloride injury to the liver takes place initially in the areas unaffected by the necrotoxic action. It could well be that preferential mitosis of mononucleate cells in the intermediate and outer zones of the lobule could lead to an absolute

increase in the number of binucleate cells seen over the remainder of the experimental period.

Whatever the nature of the response following carbon tetrachloride damage to the neonate liver, it would seem from experiments thus far that the binucleate population develops prematurely due to the increased number of cells which enter mitosis. This latter phenomenon is consequential to the necrotoxic action of carbon tetrachloride.

Having demonstrated that a necrotoxic dose of carbon tetrachloride could not induce nuclear ploidy shifts in the absence of binucleate cells, the following three experiments were carried out with a view to investigating the nature of the action of this chemical on the liver of the weanling rat. Very significant numbers of binucleate cells are present in the livers of 3-week old rats and shifts in ploidy involving a binucleate cell mechanism may thus be more easily evaluated at this age.

II. Weanling studies

Experiment 9.4

An investigation into the mitotic activity of hepatocytes after a single necrotoxic dose of carbon tetrachloride to 28-day old rats.

Method

Litters of Ash-Wistar rats were weaned at 21 days of age. On the 28th day of life the animals were dosed at 10 a.m. with 0.1 ml carbon tetrachloride/kg body weight or 20 μ l physiological saline by intra-

peritoneal injection. The animals were killed at 1 p.m. on the same day or on each subsequent day up to the fourth day of the experiment. Each rat received colcemid three hours prior to death. Four animals were represented in the control and dosed groups respectively at each time interval with sexes equally distributed within each group. After the animal had been killed, portions of liver were taken, processed, and appraised for mitotic activity as described on page 91.

Results

In Table 9.4 are set out the mean percentage mitotic counts for the dosed and control groups at each time interval (Table 9 in the Appendix gives the individual observations). The results of this experiment are also illustrated graphically in Text-Fig. 9.1b.

Statistical analysis:

An analysis of variance on the observations showed there to be a significant difference between the means of treated and control groups.

$$F = 12.66 \quad n_1 = 8 \quad n_2 = 27 \quad p < 0.001$$

Histological description

Liver sections from the control animals showed that by 28 days of age there was a definite lobular pattern with parenchymal cell cords radiating from central veins. Haemopoietic tissue was almost entirely absent, apart from the very occasional small group of erythroblastic cells.

Table 9.4

Mean and standard deviation of nuclei in metaphase expressed as a percentage of all nuclei counted in groups of Ash-Wistar rats dosed at 28 days of age and killed at varying times thereafter.

Time between CCl ₄ or NaCl administration and injection of colcemid (animals killed 3 hrs. after colcemid inj.)	Dosage regimen	
	Control group (20 μ l saline i/p)	Treated group (0.1 ml CCl ₄ /kg body weight)
0 hours	0.18 \pm 0.07	
1 day	0.23 \pm 0.32	1.32 \pm 0.95
2 days	0.26 \pm 0.22	1.57 \pm 0.30
3 days	0.27 \pm 0.17	0.68 \pm 0.31
4 days	0.12 \pm 0.10	0.15 \pm 0.07

3000 nuclei scored/liver sample.

4 animals in each group.

The lesions which carbon tetrachloride induced at the 0.1 ml/kg body weight dose level were very similar in extent to those induced in the neonate liver at the same dosage. One day after treatment a small region of degenerating parenchymal cells had developed centrilobularly and this was surrounded by a typical broad zone of cells undergoing hydropic degeneration (Fig. 9.1). The hepatocytes in the remainder of the lobule were moderately vacuolated at this time. By the second day of the experiment, active repair was in progress with removal of necrotic debris by histiocytes. Livers of the rats killed on day three showed that the residual effect of carbon tetrachloride was represented by only the occasional balloon and necrotic cell associated with central veins. Hepatocytes involved in the reparative process in areas which had previously been necrotic differed from the remainder of the hepatocyte population in that their cytoplasm was more homogeneously basophilic (Fig. 9.2). By day four the liver parenchyma had been restored (Fig. 9.3).

With specific regard to the formation of polyploid nuclei through intermediate binucleate cells, no evidence of fusion of nuclei was observed. In the livers of the 1- and 2-day treated groups, however, occasional binucleate cells with both nuclei in simultaneous prophase were seen (Fig. 9.4) and relatively large metaphase mitotic figures were also noted (Fig. 9.5).

In 28-day old treated rats examined either one or two days after dosage it was seen that the majority of mitoses occurred in the intermediate zone of the lobule (Fig. 9.6).

Discussion

It is sufficient at this point simply to note that carbon tetrachloride induces a reparative increase in the frequency of mitoses in the four week old rat liver and this increase persists for three to four days.

Experiment 9.5

An investigation into the proportion of binucleate cells in the 28-day old rats following a single necrotoxic injection of carbon tetrachloride.

Method

Litters of rats were weaned at 21 days of age. At 28 days of age they were injected intraperitoneally at 10 a.m. with 0.1 ml carbon tetrachloride/kg or 20 μ l physiological saline and killed at daily intervals up to the fourth day following the initial injection. An additional control group was included at 28 days of age. This procedure was repeated as the litters became available until there were two male and two female rats represented in each group at each time interval. Once the animals had been killed the liver samples were disaggregated and the mono- and binucleate cell population appraised as described on p 98. A total of 1000 cells was counted for each liver sample.

Results

Table 9.5 shows the mean and standard deviation of the percentage of binucleate cells found at each sampling time in the treated and

Table 9.5

Mean proportions of binucleate cells found in livers of control and carbon tetrachloride treated rats dosed at 28 days of age and killed at varying times thereafter.

Time interval between dosage and death (in days)	% Mean binucleate cells \pm SD	
	Control	Dosed
0	27.3 \pm 2.6	
1	29.4 \pm 3.5	17.7 \pm 1.8
2	29.6 \pm 5.1	8.2 \pm 4.8
3	26.7 \pm 2.7	7.9 \pm 3.4
4	27.6 \pm 3.3	12.2 \pm 5.5

The mean values were obtained from 4000 cells (1000 cells from each rat).

4 animals in each group.

control groups respectively (Table 10 in the Appendix shows the individual counts obtained for each animal). The proportion of binucleate cells containing individual nuclei with ploidy values above the diploid level was very small, so the two classes were pooled for subsequent presentation and analysis.

The results are illustrated graphically in Text Fig. 9.1d.

Statistical analysis:

An analysis of variance showed a significant difference between the means of treated and control groups.

$$F = 19.22 \quad n_1 = 8 \quad n_2 = 27 \quad p < 0.001$$

Discussion

Following the injection of carbon tetrachloride, the proportion of binucleate cells in the liver fell within 24 hours. The proportion of binucleate cells continued to decrease over the next 24 hours, remained at this low proportion throughout the third day, but appeared to have begun to rise on the fourth day of the experiment.

Reference to Figs. 9.1b and d shows that the decrease in the proportion of binucleate cells occurs during the period when mitotic activity is increased.

Experiment 9.6

An investigation of the proportions of nuclear ploidy classes following a single injection of carbon tetrachloride to 28-day old rats.

Method

Litters of rats were weaned at 21 days of age. At 28 days of age at 10 a.m. they were given either 0.1 ml/kg carbon tetrachloride or 20 μ l physiological saline by intraperitoneal injection and killed after four days. The dosing of the animals was arranged so that there were equal numbers of both sexes in each group. Samples of liver from each animal were disaggregated, smears prepared and stained with Feulgen-aqueous light green (p 102). One hundred nuclei from each liver sample were evaluated as to their DNA content.

Results

The results of this experiment are set out in Table 9.6. The distribution of the hepatocyte nuclei according to their DNA content is illustrated in the histograms of Text-Fig. 9.3 (One rat from the control group died on the second day of the experiment).

Statistical analysis:

A Students 't' test carried out on the proportions of hyperdiploid nuclei showed there to be a significant difference between the dosed and control group means.

$$t = 5.44 \text{ with } 13 \text{ degree of freedom } p < 0.001$$

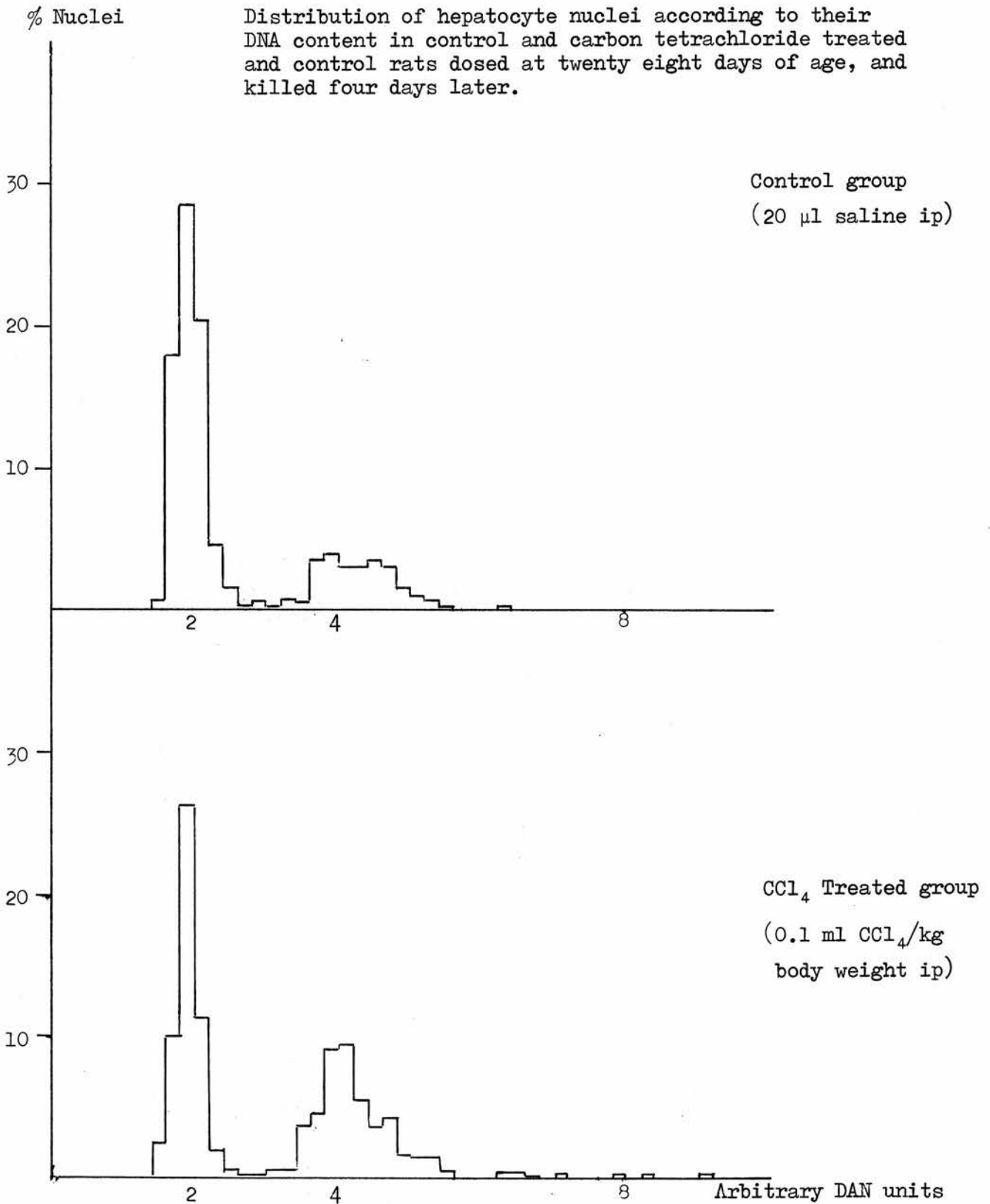
Thus, a significant shift in nuclear ploidy was induced within four days of a necrotic dose of carbon tetrachloride.

Table 9.6

Distribution of hepatocyte nuclei according to their DNA content in control and carbon tetrachloride treated groups of rats dosed at twenty eight days of age and killed four days later.

Dosage regimen	Rat No.	Sex	% 2N	% 4N	% 8N
Control group (20 μ l saline ip)	1	M	79	21	-
	2	M	65	35	-
	3	M	60	40	-
	4	M	72	28	-
	5	F	82	18	-
	6	F	80	20	-
	7	F	87	13	-
	8	F	-	-	-
	Mean		75.0	25.0	-
	SD		± 9.7	± 9.7	-
Treated group (0.1 ml CCl ₄ / kg body wt.)	1	M	51	49	-
	2	M	48	51	1
	3	M	57	42	1
	4	M	53	47	-
	5	F	58	42	-
	6	F	51	46	3
	7	F	54	46	-
	8	F	61	39	-
	Mean		54.1	45.25	0.6
	SD		± 4.2	± 4.2	-

Text-Fig. 9.3



III General discussion on Chapter 9

It is clear from the six experiments described in this chapter that nuclear enlargement following carbon tetrachloride administration involves an intermediate mitotic binucleate cell process. The carbon tetrachloride damage evokes a regenerative wave of mitosis in the liver and binucleate cells are involved in this restorative process. The events leading to the division of binucleate cells presumably involves both nuclei entering DNA synthesis and, later, mitosis simultaneously. This suggestion is well supported by observations by other workers (Beams and King, 1942; Sulkin, 1943; Himes et al., 1957; Messier and Leblond, 1960; Schultze and Oehlert, 1960; Nadal and Zajdela, 1966; Edwards and Klein, 1961; Carriere, 1967). During mitosis the chromosomes from both nuclei come together on a common metaphase plate. At the end of metaphase the net result of this process may be either a binucleate cell in interphase or two mononucleate cells, if cleavage of the cytoplasm has occurred. The ploidy levels of these nuclei, however, will be double that of the parent nuclei. This concept is further supported by my own observation of two nuclei in binucleate cells entering prophase together. On the other hand, no observations of binucleate cells with two mitotic spindles were made. The evidence from Experiment 8.5 is that, in most instances at this age, binucleate mitosis ends with cleavage of the cytoplasm and two mononucleate cells are formed. This would be the only mechanism which could account for the profound drop in the level of binucleates observed in Experiment 9.5.

Himes et al. (1957) also demonstrated that carbon tetrachloride

administration to rats of eight weeks of age and older resulted in a drop in the numbers of binucleate cells in the liver and an increase in the size of hepatocyte nuclei. These workers were also able to show that repeated carbon tetrachloride administration led to a depletion of binucleate cells until nuclear polyploid increases were no longer detectable. Thus, it would seem that this type of pathological nuclear enlargement develops in direct relationship with the number of binucleate cells present in the liver. It should be remembered, however, that the increased mitosis in the liver parenchymal cells, as induced by carbon tetrachloride damage, would presumably have some influence on the development of new binucleate cells from mononucleate cells since this also involves a mitotic process (Beames and King, 1942; Brues and Marble, 1937; Wilson and Leduc, 1942). Thus, repeated carbon tetrachloride damage could, by bringing about both formation and loss of binucleate cell numbers in the liver lead to the gradual but eventually marked increase in cell and nuclear size as observed by Eschenbrenner and Miller (1946) in the adult rat. In the experiments of this chapter, however, the shift in ploidy was from 2N to 4N only and the action of carbon tetrachloride was, as it were, to anticipate the normal rapid development of nuclear polyploidy via the binucleate population which has been reported to occur at around this age. (Alfert and Geschwind, 1958; Carriere, 1962; James et al., 1966; Nadal and Zajdela, 1966).

There are one or two other aspects of the experiments of this chapter which are worthy of further discussion with respect to the findings of other workers.

A comparison of the mitotic activity of the hepatocytes at the two ages studied (see Text-Figs. 9.1a and b) shows that by four to five weeks of age the level had fallen to approximately half that seen in the rat during the first eight days of life. This is in general agreement with other studies which have shown a similar decline after four to five weeks of age (McKellar, 1949; Tier and Ravanti, 1953; Givol, 1957; Nadal and Zajdela, 1966). Table 9.7 compares the mitotic activity in the liver found by previous workers with the results obtained in this chapter.

Table 9.7

Comparison of mitotic activity found by
previous workers at various ages in the
rat

Age in weeks	References				
	A	B	C	D	E +SD
0	1		14	1.3	0.79 ± 0.50
$\frac{1}{2}$					0.55 ± 0.14
1	12	0.21	13	2.1	0.70 ± 0.39
2	23		11	2.6	
3	33		10	4.5	
4	25	0.26	3	0.5	0.18 ± 0.07
$4\frac{1}{2}$					0.27 ± 0.17
5				2.9	
6	11	0.11	2.5	0.3	
7					
8			1.1		

- A. McKellar 1949 - Lobular index - colchicine arrest.
- B. Nadal and Zajdela 1966 - % mitoses - no colchicine.
- C. Tier and Ravanti 1963 - No mitoses/100 fields - colchicine arrest.
- D. Givol 1957 - % mitosis - colchicine arrest.
- E. Own work - % mitoses - colcemid arrest.

Close comparisons of mitotic activity are impossible. Some workers used colchicine over varying time intervals whilst others did not and in any event different methods of enumeration were employed. Nevertheless, it is clear that a fall in the mitotic activity of parenchymal cells occurs between the fourth and fifth week of life. The results from the work of McKellar (1949) over the first three weeks differ to some extent from the remainder in that he detected a sharp increase in mitoses at around two weeks of age. This disagreement might be attributable to the fact that McKellar's observations were on one or two animals only at each time interval. The work of this thesis on mitotic activity of the rat up to one week of age, as previously mentioned, has shown the considerable individual variation that can occur, and emphasised the need for the use of sufficient numbers of animals in experiments of this nature. This point is further stressed by results on the mitotic activity in the livers of adult rats reported by Carriere (1969) where individual animals within groups often had mitotic indices 25 or more times higher than the remaining rats. This worker could not detect any valid reason for eliminating the high values from these experiments.

The time intervals studied in experiments of this thesis were too far apart to enable one to draw any conclusions with regard to when the peak in mitotic activity was reached at either age. Following partial hepatectomy in the adult rat other workers have found that mitotic activity in the hepatocyte population commences 20-24 hours after the operation to reach a maximum at around 28 hours, after which the activity falls off rapidly at first and then slowly over the next few days, (Brues and Marble, 1937; Cater et al., 1956; 1957; Harkness, 1957; Butcher, 1963). On this basis it is apparent that the approximate peak of mitotic activity was almost certainly missed in the appropriate experimental results of this chapter.

One feature of this regenerative response to carbon tetrachloride damage which is of interest is the relative speed of healing of the liver at the two ages studied. It will be recalled that regeneration, as evaluated by mitotic activity and histological restoration following carbon tetrachloride poisoning of the four-day old rat, was complete by the third day following administration. In the 4-week old rat, however, the hepatocyte changes of hydropic degeneration and necrosis could still be seen at this time; furthermore mitotic activity in the livers of the treated animals was still at a significantly higher level than in control livers on the third day following administration (difference between means of control and treated groups on day three tested for with Student's 't' test, $p < 0.05$). A further indication that regeneration is more rapid in the younger rats comes from a comparison of Figs. 9.1a and 9.1b. These figures show that, in the 4-day old rat administered carbon tetrachloride, there was a much higher level of

mitotic activity on the first and second days of the experiment than at the corresponding times in the weanling rat, even though the lesions induced by carbon tetrachloride in both age groups were very similar in extent.

It is relevant to this finding, that Post et al. (1960) in studying the healing rate of the liver following the administration of carbon tetrachloride to rats of ages varying between three weeks and two years, found that repair, as evidenced by resolution of the necrotic zone, took only three days in the 3-week old rat but was not complete after as long as five days in the two year old group of rats studied. These workers also evaluated mitotic activity in the liver and observed that this too returned to control levels at a faster rate in the young animals. Earlier work by Post et al. (1957) had shown that regeneration in terms of mitotic activity, polyploidy and architectural restoration following carbon tetrachloride administration was markedly exaggerated by administration of growth hormone. The relatively greater speed of regeneration in the young growing rat was attributed by Post et al. (1960) to the possibility of greater influence of this hormone in early life.

The rapid rate of healing in the young rat might be taken to indicate that the replicating times of hepatocytes could be of shorter duration than in older animals. This aspect of hepatocellular kinetics was studied by Post and Hoffman (1964) using tritium labelled thymidine for a study of time phase changes of nuclear interphase and mitotic labelling. They found the average replication time for hepatocytes in 1-day old rats to be almost half that which was found in 3-week old

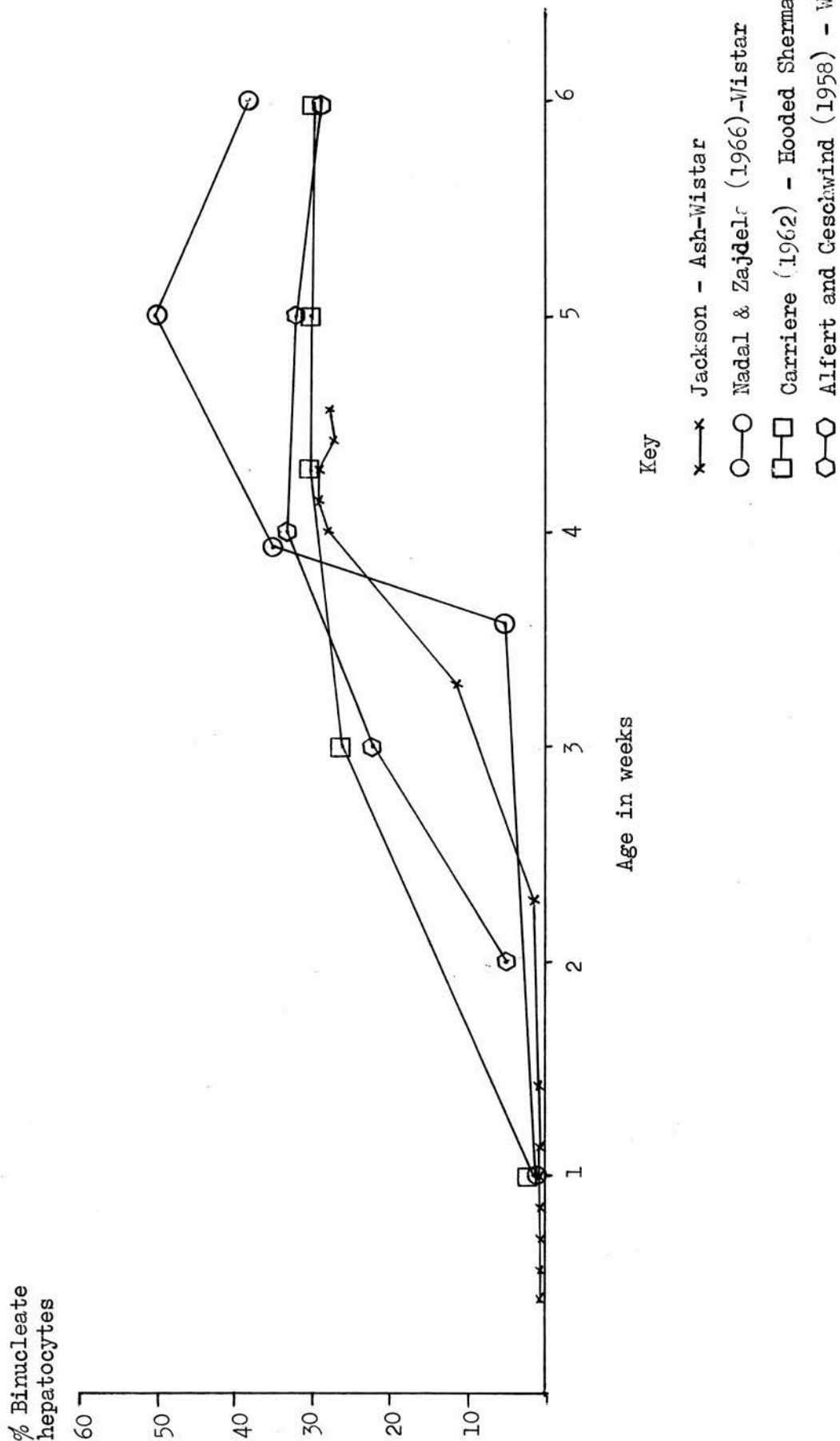
rats and which in turn was approximately half that found in the 8-week old animals. Once again it was suggested that the accelerated repair seen with growth hormone (Post et al. 1957) and the reverse effect produced by cortisone indicated that the antagonistic action of these two hormones might play a part in regulation of the cell cycle during growth and regeneration.

Such a concept is supported by other work since anterior pituitary growth hormone has been shown to be necessary to maintain the normal growth rate of the liver (Simpson et al., 1949). Furthermore, injection of growth hormone into intact mice has been shown to stimulate parenchymal cell mitosis (Litman et al., 1958) while hypophysectomy prior to partial hepatectomy in the rat results in a delayed onset of regeneration (Higgins and Ingle, 1939; Rabes et al., 1956; Hemmingway and Cater, 1958).

Turning to the physiological accumulation of binucleate cells in the liver, Text-Fig.9.4 compares the pooled results of appropriate experiments of this thesis with the findings of other workers. It may be seen that, although there is marked variation between results by respective authors, the general trend is for an increase of binucleate cell numbers to begin at the end of the second week of life; the highest proportions of binucleate cells are attained between the third and the fifth weeks of life. Carriere (1969) ascribed this difference in results from separate laboratories to variations in the growth rate of the liver in the different strains of rats used. Here again anterior pituitary growth hormone would appear to play an important role in the early accumulation of binucleate hepatocytes since they are found to be

Text-Fig. 2.4

Comparison of accumulation of binucleate cells in the rat liver over the first six weeks of life.



almost entirely absent in mice suffering from hereditary anterior pituitary dwarfism (Helweg-Larsen, 1952). Injection of anterior pituitary growth hormone into these mice resulted in the development of a binucleate hepatocyte population.

Finally, mention should be made of the accumulation of nuclear polyploidy in the liver as seen in the appropriate experiments described up to this point. Other workers (Alfert and Geschwind, 1958; Carriere, 1962; Nadal and Zajdela, 1966) are in general agreement with these results, in that the nuclear population is almost exclusively diploid up to three weeks of age, after which there is a sharp rise in tetraploid nuclei. Experiment 9.4 showed that by day thirty-two of life a rapid rise in tetraploid nuclei had occurred to reach a level of 25%. Once again, growth hormone would appear to have a pronounced effect on nuclear polyploid development. Two examples of the many experiments which could be cited in support of this suggestion will be given. Anterior-pituitary dwarf-mice fail to develop nuclear polyploidy to any marked degree (Helweg-Larsen, 1952) while marked increases in nuclear polyploidy are seen almost immediately after injection of anterior growth hormone into these animals. Administration of growth hormone to adult hypophysectomised rats also causes increased nuclear polyploidy (Geschwind et al., 1958) as compared to appropriate controls.

The main conclusions which may be drawn from the experimental work of this chapter are as follows. In order to produce an increase in nuclear ploidy, the increases in mitotic activity induced by carbon tetrachloride must be initiated at an age when significant numbers of binucleate cells have accumulated in the liver. This ploidy increase

requires that both the nuclei in binucleate hepatocytes enter mitosis simultaneously and form a common metaphase plate. In most instances, two mononucleate cells of the next highest ploidy level are then formed.

It has also been seen that the healing of the liver is very rapid in the neonate rat and becomes a more prolonged process as the animals age. The influence of anterior pituitary growth hormone on this aspect of liver regeneration has been discussed.

Two mechanisms for the induction of increased nuclear ploidy have been demonstrated thus far, one for DMN and one for carbon tetrachloride. The next chapter briefly investigates the manner in which a third hepatotoxic chemical, thiocetamide, brings about this effect.

Illustrations

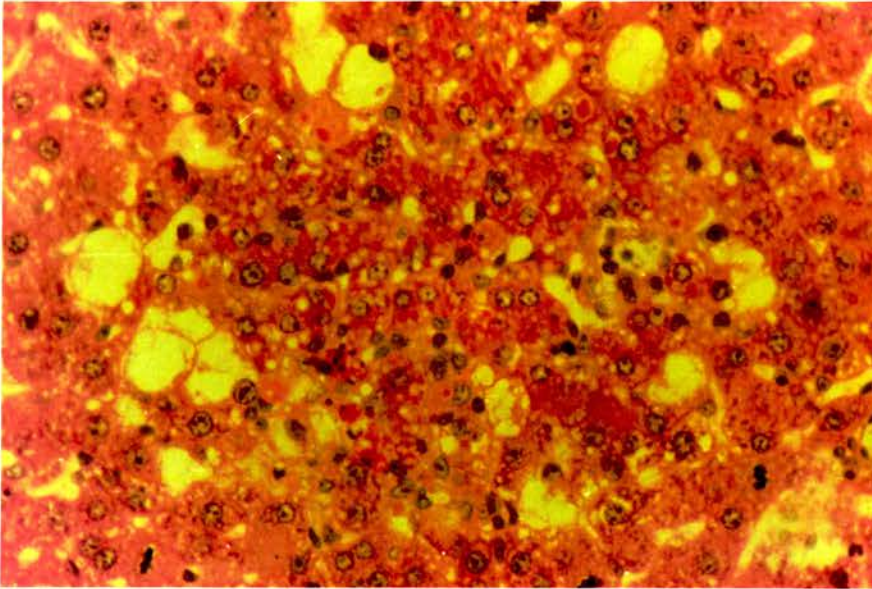


Fig. 9.1 Liver from a 29 day-old rat injected with 0.1 ml carbon tetrachloride 27 hours before, and colcemid 3 hours before being killed. The central zone of necrosis is surrounded by the typical 'balloon' cells undergoing hydropic degeneration. Many mitotic figures may be seen in the surrounding liver parenchyma. (H+E x 220)

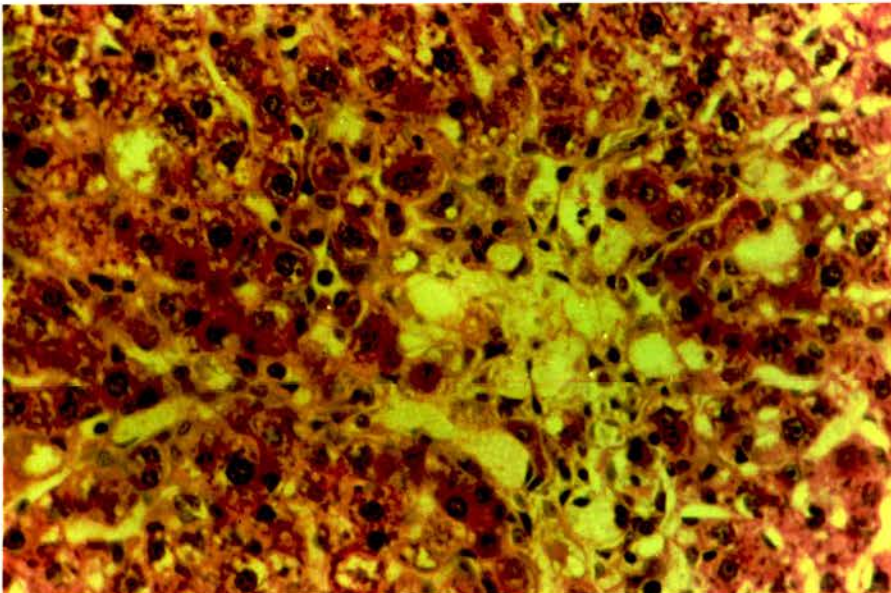


Fig. 9.2 Liver from rat treated as above but killed at 3 days following the injection of carbon tetrachloride. There had been considerable resolution of the lesion by this time. (H+E x 220)

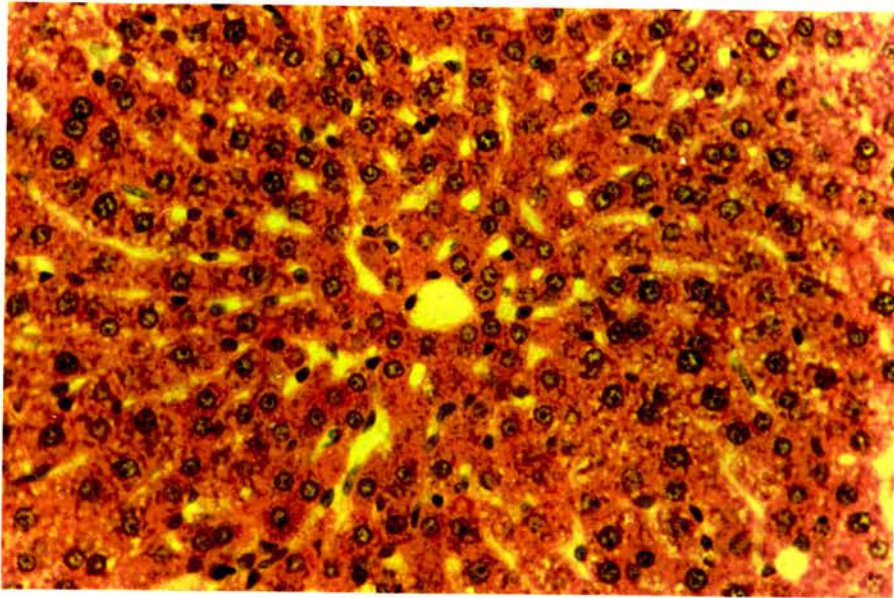


Fig. 9.3 By the fourth day following the injection of 0.1 ml carbon tetrachloride/kg body weight the restoration of the liver parenchyma was complete. (H+E x 220)

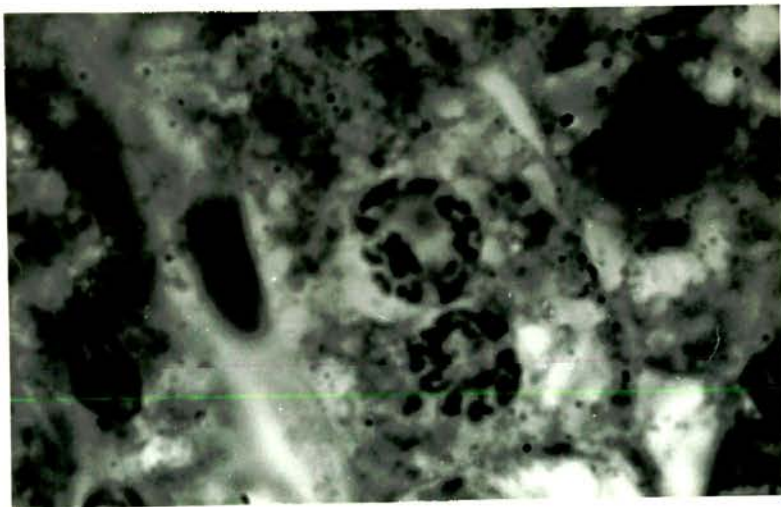


Fig. 9.4 Liver of animal treated with 0.1 ml carbon tetrachloride/kg body weight 48 hours previously and showing a binucleate hepatocyte with both nuclei in simultaneous prophase. (H+E x 2200)

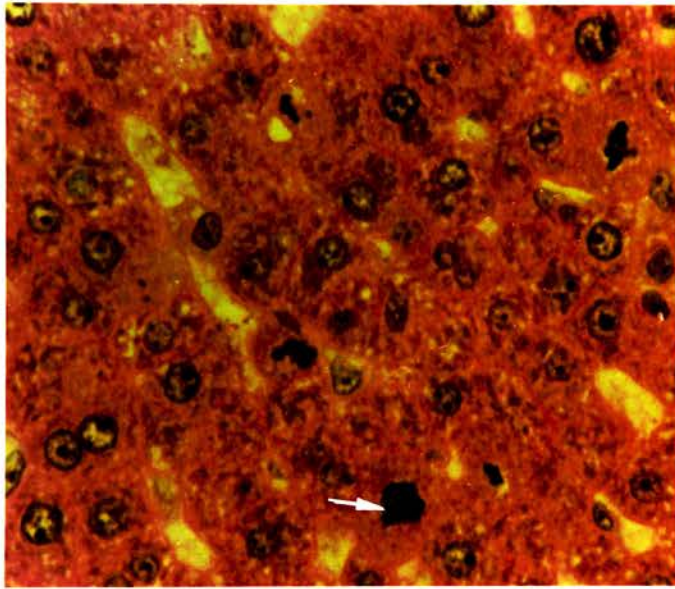


Fig. 9.5 Liver from a rat treated two days previously with 0.1 ml carbon tetrachloride/kg body weight. A relatively large metaphase mitotic figure is seen arrowed which in the text is suggested to have arisen from simultaneous mitosis of the two nuclei in a binucleate cell. (H+E x 550)

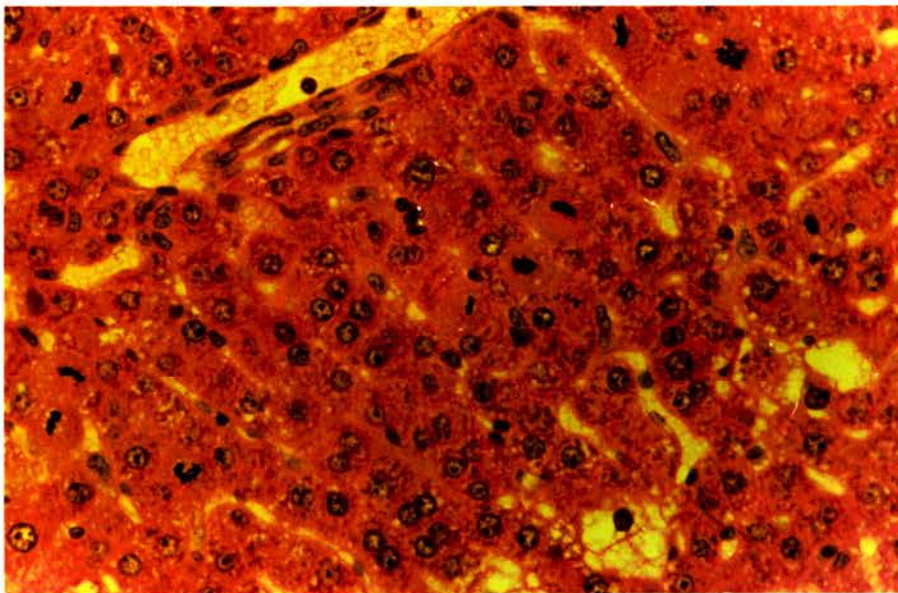


Fig.9.6 Liver from 29 day-old rat treated 24 hours previously with 0.1 ml carbon tetrachloride/kg body weight and which had received colcemid treatment. The intermediate zone of the lobule contained the majority of mitotic figures in most instances. (H+E x 360)

CHAPTER 10

THE NATURE OF NUCLEAR ENLARGEMENT FOLLOWING
THE ADMINISTRATION OF THIOACETAMIDE

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It has been established thus far that carbon tetrachloride induces increases in nuclear ploidy through an intermediate binucleate cell system whereas DMN acts through a block in the cell cycle following DNA synthesis. At this point in the work it was thought that it would be of interest to briefly investigate the way in which a third hepatotoxin brings about increased nuclear ploidy. Thiocetamide has been shown to induce such an effect in the liver of the rat (Rather, 1951; Bernstein et al., 1953; Hiezer, 1955; Kleinfeld, 1957) but the way in which this compound brings about nuclear enlargement has not been elucidated. Four experiments were accordingly carried out to investigate the nature of the increase in nuclear size.

In these experiments a wave of mitosis was demonstrated following administration of the compound to the four-day old rat but no shift in ploidy occurred at this time. In the weanling rat however, treatment with thiocetamide resulted in a marked fall in the level of binucleate cells with an associated increase in nuclear ploidy. On this evidence it was concluded that thiocetamide brings about increases in nuclear ploidy in the same manner as carbon tetrachloride; that is, through an intermediate binucleate cell system.

1. Studies with 4-day old rats

Experiment 10.1

An investigation of mitotic activity and nuclear ploidy of parenchymal cells following a single administration of thiocetamide to the neonate rat.

Method

Two litters of Ash-Wistar rats were dosed at four days of age at 10 a.m. with 100 mg thiocetamide/kg body weight or 20 μ l physiological saline by intraperitoneal injection. Dosing was arranged so that there were at least four animals in the control and treated groups in each litter. Twenty-four hours later the rats of litter 1 received colcemid treatment and were killed after a further three hours. The rats of litter 2 received colcemid treatment 42 hours after the thiocetamide treatment and likewise were killed after a further three hours. The liver of each animal was processed and appraised for mitotic activity as has previously been described (p 91).

A third litter of eight animals, also four days of age, were dosed with thiocetamide or physiological saline as above. This litter was killed four days after these injections. Liver samples from each animal were disaggregated and smears prepared and stained Feulgen aqueous-light green. The DNA content of 100 parenchymal cell nuclei was measured from each liver sample. Small portions of liver were also taken for histology, embedded routinely in paraffin wax, sections cut at 4 μ and stained Ehrlichs alum haematoxylin and eosin.

Results

i) Mitotic activity

Table 10.1 shows the observed mitotic counts in the individual liver samples.

A students 't' test carried out on the means of the groups at each of the time intervals failed to detect any significant difference at the 24-27 hour interval ($p > 0.05$). The means of the treated and control

Table 10.1

Percentage of metaphase mitotic figures counted
in Ash-Wistar rats dosed at 4 days of age and
killed at varying times thereafter.

Time interval between administration of thiocetamide and inj. of colcemid (animals killed 3 hrs. after colcement treatment)	Dosage regimen			
	Control group (20 μ l saline i/p)		Treated group (100 mg thiocetamide /kg body wt)	
	Sex	% metaphase mitotic figures	Sex	% metaphase mitotic figures
24 hours	F	0.33	F	0.70
	M	0.63	F	0.96
	M	0.53	M	1.20
	M	0.86	M	1.26
	Mean \pm SD	0.59 \pm 0.22		1.03 \pm 0.26
42 hours	F	0.59	F	2.27
	F	1.20	F	3.03
	M	0.60	F	2.80
	M	0.86	M	2.60
	Mean \pm SD	0.31 \pm 0.29		2.68 \pm 0.32

groups killed at forty-five hours were significantly different.

$t = 7.51$ with 6 degrees of freedom $p < 0.001$

The histological appearance of the control livers was as has been described previously (p 136) in the neonate rat at this age.

The livers of the animals dosed with thiocetamide and killed after twenty seven hours showed small areas of centrilobular change in hepatocytes, uniformly distributed throughout the liver sections. These areas showed a minimal loss of hepatocytes at this time but many parenchymal cells had swollen, very pale nuclei and markedly vacuolated cytoplasm. There was also a marked infiltration of this area by neutrophil polymorpholeukocytes. The appearance of the centrilobular zone at this time interval may be seen in Fig. 10.1. Nuclei of hepatocytes in the remaining areas of the liver contained enlarged prominent nucleoli. The structures of portal tracts were normal in appearance and the haemopoietic tissue was unaffected by the treatment.

By the second day following the injection of thiocetamide the only feature centrilobularly was the occasional hepatocyte with a pale swollen nucleus, and an obvious increase in mitosis in the remainder of the liver. The mitotic activity was mainly confined to the mid-zonal and periportal zones.

Histological examination of the livers from the treated rats used for the ploidy estimation showed that by day 4 this centrilobular lesion had been completely resolved. The only detectable difference from control animals was the increase in the size of nucleoli.

ii) Ploidy evaluation

Table 10.2 below sets out the relative proportions of 2N and 4N nuclei in the treated and control groups (No hypertetraploid nuclei were observed in either group).

Table 10.2

Percentages of the hepatocyte nuclei in each ploidy class in Ash-Wistar rats dosed at 4 days of age and killed four days later

Dosage regimen	Rat No.	Sex	% 2N	% 4N
Control group (20 µl saline i/p)	1	M	93	7
	2	M	93	7
	3	F	99	1
	4	F	96	4
	Mean		95.25	4.75 \pm 2.87 (\pm SD)
Treated group (100 mg thio- cetamide/kg body weight)	1	M	92	8
	2	M	92	8
	3	M	95	5
	4	F	93	7
	Mean		93	7.0 \pm 1.41 (\pm SD)

No statistically significant difference was detected between the two means and it was concluded that the administration of thiocetamide, although inducing a wave of mitosis at this level, had no significant effect on nuclear ploidy in the liver of the neonate rat.

2. Weanling studies

Experiment 10.2

An investigation of nuclear ploidy and proportions of binucleate cells in the weanling rat following a single dose of 100 mg thiocetamide/kg body weight.

In Experiment 9.5 involving administration of carbon tetrachloride to the weanling rat it was seen that the control groups had already developed a significant population of tetraploid nuclei by thirty-two days of age. The litter in this experiment was therefore dosed at a slightly earlier age in an attempt to obtain control observations before this ploidy development had taken place.

Method

A litter of eight Ash-Wistar rats were dosed on the twenty-fourth day of life with 100 mg thiocetamide/kg body weight or with 20 µl physiological saline by intraperitoneal injection. Dosing was arranged so that there were four animals in each group. The litter was killed four days later and the livers disaggregated. Smears were prepared for Feulgen microspectrophotometry (100 hepatocyte nuclei measured/liver sample) and counts of binucleate cells were made in an haemocytometer as previously described (p 98). Portions of liver from each animal were also processed and embedded in paraffin wax and sections cut and stained with Ehrlich's alum haematoxylin and eosin.

Results

Table 10.3 shows the proportions of tetraploid nuclei and binucleate cells observed in the liver samples (The numbers of binucleate

cells containing two hyperdiploid nuclei were so few in both groups that they were pooled with the binucleate cells containing two diploid nuclei for subsequent analysis. The numbers of these binucleate cells containing hyperdiploid nuclei observed in any count are shown in parentheses where appropriate).

A students 't' test carried out on these respective sets of figures demonstrated a significant difference between the means of binucleate cells; $t = 3.30$ with 6 df $p < 0.02$ and a significant difference between the means of the tetraploid nuclei in the two groups;

$t = 4.16$ with 6 df $p < 0.01$.

Text-Fig. 10.1 shows the actual distribution of the ploidy populations.

Histological appearance

Microscopical examination of the livers of the weanling animals treated with thiocetamide four days previously failed to reveal any gross abnormalities. Nuclear size exhibited more variation and, as with the neonate livers, the nucleoli appeared to be enlarged when compared to the control livers. No abnormalities of the bile ducts or blood vessels could be discerned.

Table 10.3

Percentages of binucleate cells and tetraploid nuclei
in Ash-Wistar rats dosed at 24 days of age and killed
when 28 days old.

Dosage regimen	Rat No.	Sex	% Binucleate cells	% Tetraploid nuclei
Control group (20 μ l saline i/p)	1	F	26.9	10
	2	F	31.5 ⁽¹⁾	2
	3	F	29.9	8
	4	M	38.3	7
	Mean \pm SD		31.7 \pm 4.8	6.75 \pm 3.4
Treated group (100 thiocetamide /kg body wt)	1	F	13.1 ⁽²⁾	49
	2	M	11.5	48
	3	F	25.3	15
	4	F	11.8 ⁽¹⁾	35
	Mean \pm SD		15.4 \pm 6.6	36.75 \pm 15.8

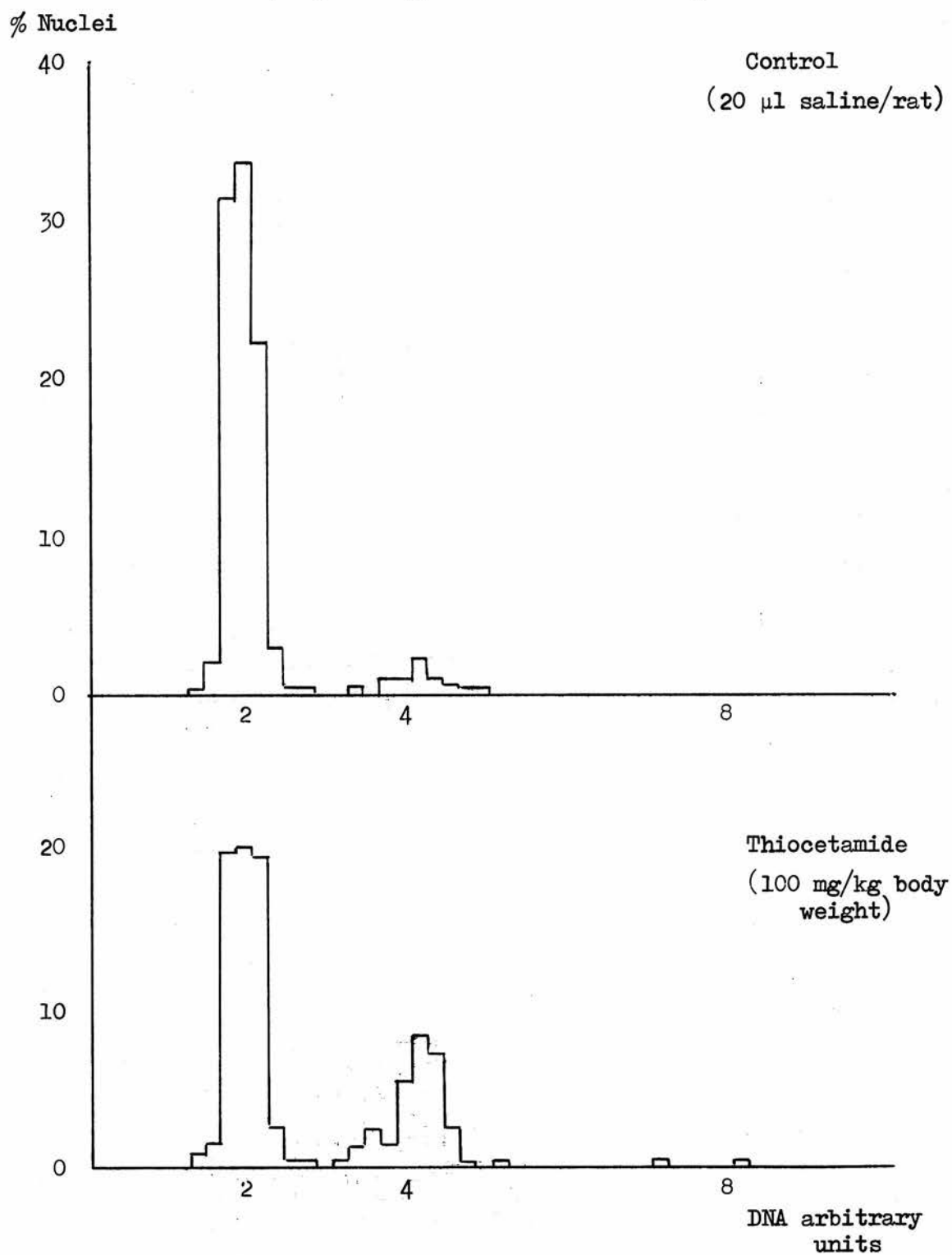
1000 hepatocytes
scored/liver
sample

100 hepatocyte
nuclei scored/
liver sample

Values in parenthesis indicate the number of binucleate
cells containing two hyperdiploid nuclei.

Text-Fig. 10.1

Ploidy distribution of hepatocyte nuclei in control and thiocetamide treated rats injected at 4 days of age and killed four days later.



Discussion

Thiocetamide failed to give rise to increased nuclear ploidy when given at a necrotoxic level to four-day old rats at which age there are negligible numbers of binucleate cells. On the other hand administration to weanlings resulted in a marked shift in nuclear ploidy with an associated fall in the numbers of binucleate cells. On the above evidence it is concluded that thiocetamide increases nuclear ploidy in the liver via binucleate cell division as discussed fully with carbon tetrachloride in the previous chapter.

Nuclear enlargement following thiocetamide administration has been investigated by a number of experimentalists in the past. The mechanism of the increases in ploidy has not been previously established although it is well known that increase in nuclear size due to thiocetamide involves an increase in nuclear DNA content (Kleinfeld, 1957; Hiezer, 1955; Bernstein et al., 1963). The experiments of Bernstein et al. (1953), however, which were concerned with measuring hepatocyte nuclear diameters following thiocetamide administration, indicated that this increase in size was not exclusively due to increase in DNA content. These workers based their argument on the fact that nuclear size determinations did not conform closely to a geometric series (as occurs in normal liver). The view that another nuclear constituent, in addition to DNA, was involved in the nuclear enlargement was further strengthened by the photometric and volume measurements of Kleinfeld (1957). This worker found that withdrawal of thiocetamide administration resulted, after a period of four days, in a decrease in nuclear volume though the increased nuclear polyploid values remained constant. The

most typical and conspicuous change that ensues after thiocetamide involves the structure and metabolism of the nucleolus which leads to an increase in RNA content (Fitzhugh and Nelson, 1948; Steele et al. 1965; Godwin et al. 1967; Kleinfeld, 1957). Nucleolar changes would therefore appear to be involved to some extent at least, in increases in nuclear volume which are unassociated with increased DNA content.

The cellular kinetics in the liver following thiocetamide administration were very poorly understood until the recent experiments of Reddy et al. (1969) were published. It is interesting that these authors reported finding an increase in both DNA synthesis and mitosis in the adult rat seemingly below a level of administration required to induce necrosis (50 mg/kg). This is essentially in agreement with the experiment of this chapter where only very slight degenerative changes of the neonate liver were produced by 27 hours when this compound was administered at 100 mg/kg. Nevertheless a marked increase in mitotic activity was seen by 42 hours after injection. It is thought that this mitotic wave in the neonate liver was excessive when viewed in the light of the minimal damage brought about by thiocetamide at this level of administration. In this respect it is interesting to compare the respective mitotic activities induced in the neonate liver by carbon tetrachloride and thiocetamide (see Tables 9.1 and 10.1) since the former chemical had increased division of parenchymal cells very considerably by 24 hours following injection. With thiocetamide on the other hand, the increase in mitotic activity was not seen until the second day following administration. With respect to this latter

feature Reddy et al. (1969) found the maximum mitotic rate in the adult rat to be between forty and fifty hours following intraperitoneal injection. The inflammatory response to the thiocetamide induced necrosis also varied markedly from that of carbon tetrachloride and DMN found in the neonate, it consisting of large numbers of neutrophil polymorphonuclear leukocytes (see Fig. 10.1). Gupta (1956) reported the acute histological lesion in the adult rat liver following a single subcutaneous 200 mg/kg injection. At this level Gupta observed necrosis by 24 hours following administration and this developed further over the ensuing twelve hours. Most of the necrotic cells had been removed by 48 hours and by the fifth day the liver was completely healed.

It would seem from this that overt necrosis from thiocetamide treatment is possible if the compound is given at sufficiently high levels. It would also seem however that this liver toxin is capable of initiating an increase in mitotic activity which is unrelated to its necrotoxic action. Reddy et al. (1969) suggested that the nucleolar changes observed with the compound might be involved in this effect.

ILLUSTRATION

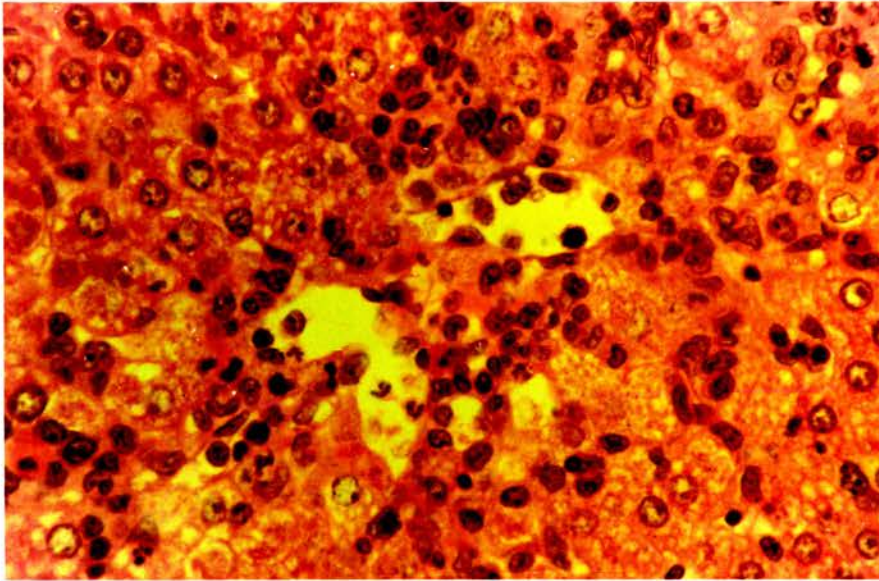


Fig. 10.1 Liver of rat which received 100 mg thiocetamide/kg body weight 24 hours previously. Many of the hepatocytes in the centrilobular region have pale swollen nuclei and vacuolated cytoplasm. There is also a gross infiltration of this area by polymorpholeukocytes.

CHAPTER 11

FURTHER INVESTIGATIONS INTO THE NATURE
OF THE ANTIMITOTIC EFFECT BROUGHT ABOUT
BY DIMETHYLNITROSAMINE IN THE LIVER

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The experiments in Chapter 8 described the manner in which DMN brings about increased nuclear ploidy in the liver by blocking the entrance of cells into mitosis following their completion of DNA synthesis. Moreover, the single dose of DMN would appear to exert this effect on one cell cycle only, thus inducing a single rise to the next nuclear ploidy level in those hepatocytes affected.

The aims of the experiments described in this chapter were to investigate:-

i) Whether the number of hepatocytes blocked and the subsequent increase in ploidy whilst the antimitotic effect of DMN is present, is a function of the number of cells entering DNA synthesis.

ii) The duration of the antimitotic effect of DMN following single dosage.

iii) Whether extreme cell and nuclear enlargement can be induced by continued DMN administration so that cells are repeatedly prevented from entering mitosis during successive cell cycles with the development of megalocytosis of hepatocytes.

Experiment 11.1 - To investigate the extent to which the increased nuclear ploidy is dependent on the number of cells stimulated to enter DNA synthesis

It was seen in Chapter 9 that carbon tetrachloride had no effect on the nuclear ploidy state of the neonate rat liver following a wave of mitosis induced by this compound. It was therefore considered valid to use carbon tetrachloride in order to produce a regenerative wave of mitosis during the time that DMN was exerting its antimitotic effect in an attempt to show that the ploidy increase was dependent on the number

of cells stimulated to enter DNA synthesis.

Method

A litter of 8, 4-day old rats was injected intraperitoneally as follows:-

- Regimen I Four rats with simultaneous injections of 0.1 ml carbon tetrachloride/kg body weight and 10 mg dimethylnitrosamine/kg body weight.
- Regimen II Two rats with 10 mg dimethylnitrosamine/kg body weight.
- Regimen III One rat with 0.1 ml carbon tetrachloride/kg body weight.
- Regimen IV One rat with 20 μ l physiological saline.

The survivors of these treatments were killed four days later. Feulgen stained smears of the liver from each animal were prepared and appraised as described (p 102). Portions of each liver were also fixed in 10% neutral buffered formalin, routinely processed and embedded in paraffin wax. 4 μ sections were cut and stained by Ehrlich's alum haematoxylin and eosin.

Results

Three of the rats administered the simultaneous injection of CCl_4 and DMN were cannibalised within 24 hours of administration (a previous attempt at this experiment was abandoned due to the cannibalisation of all the rats in this group, presumably following death). The sole survivor was grossly emaciated and weighed only 6.44 grams as compared to the control rats which weighed 17.80 grams. The ratios of liver weight to body weight expressed as a percentage were 5.16% and 2.97% respectively in the DMN + CCl_4 treated and control animals.

Macroscopic examination of the survivor of Regimen I showed the liver to be pale with a mottled appearance of the surface and cut surface of all lobes. It had a firm consistency and adhesions were present between the liver and viscera. The animals of the remaining groups appeared normal on macroscopic examination.

The percentage ploidy classes found in each group were as under and as shown in histogram form in Fig. 11.1

Table 11.1

The percentages of nuclei in the various ploidy classes in livers of Ash-Wistar rats which had been treated at four days of age and killed four days later.

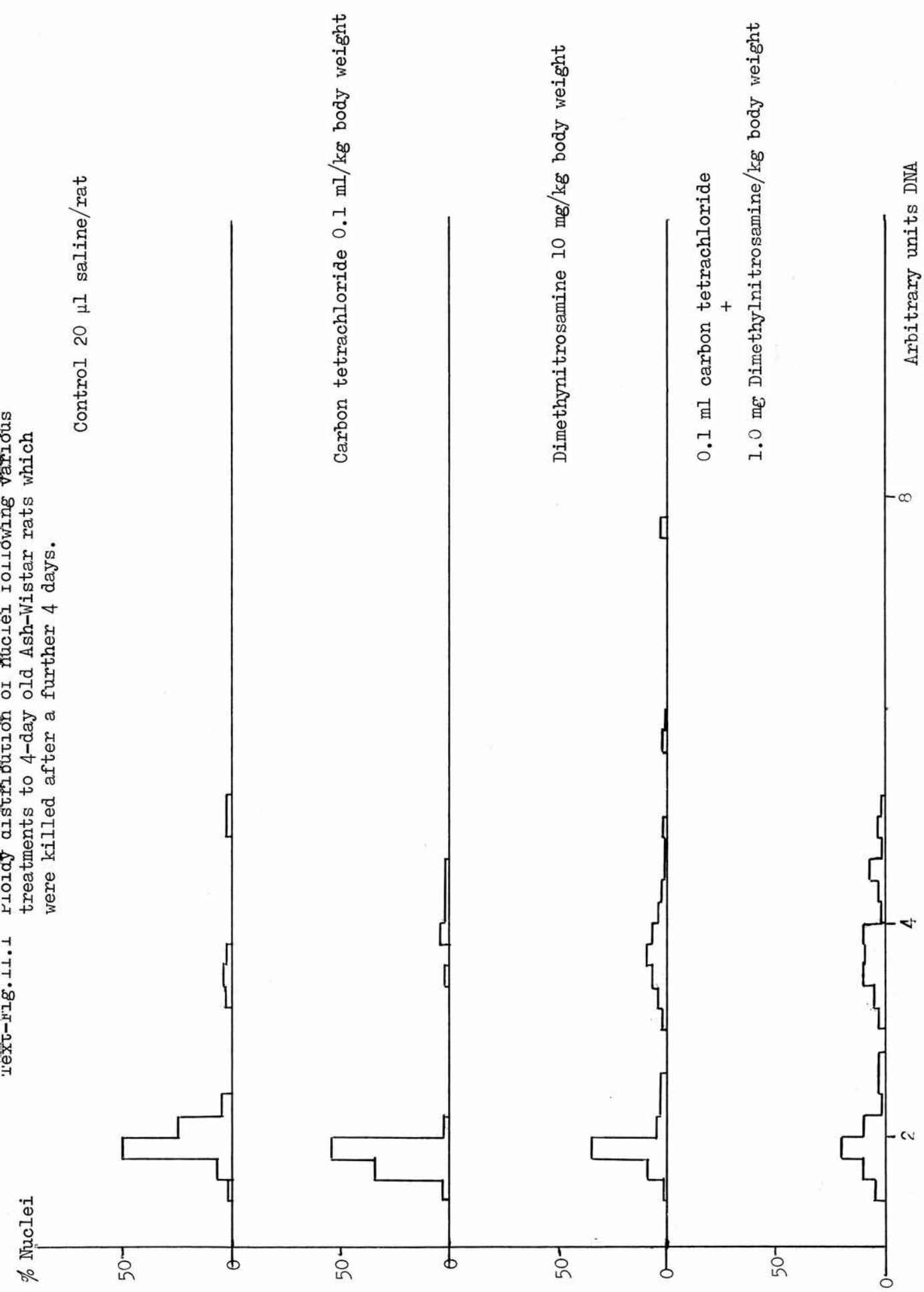
Group	Dosage regimen	%2N	%4N	%8N
I	10 mg DMN + 0.1 ml CCl ₄ /kg body weight	46	54	-
II	DMN 10 mg/kg body weight	60	39	1
III	CCl ₄ 0.1 ml/kg body weight	93	7	-
IV	Control 20 µl saline /rat	94	6	-

(One rat in each group except Regimen II in which there were two rats. The values in this group are the means of ploidy classes observed in 100 nuclei from each liver sample.)

Histology

Microscopic examination of the liver of the control animal administered physiological saline failed to reveal any abnormalities. It has been described before how the liver of the neonate rat recovers

text-fig.11.1 Fluorescence distribution of nuclei following various treatments to 4-day old Ash-Wistar rats which were killed after a further 4 days.



completely within four days of the administration of carbon tetrachloride and similar observations were made in this experiment. The livers of the animals treated with DMN presented an appearance which has already been described in Chapter 6.

By comparison, the liver of the animal which survived the simultaneous injection of DMN and carbon tetrachloride exhibited greater numbers of enlarged hepatocyte nuclei than did the livers of those rats given DMN alone. Whilst the general architecture of the liver was restored by the fourth day a number of Kupffer cells in the midzonal and centrilobular areas were grossly swollen and in many instances contained cell debris. Some cell and nuclear debris not taken up by Kupffer cells was also observed in a few centrilobular areas at this time. The bile ducts and blood vessels in the portal tracts appeared normal and no differences were noted in the mitotic activity of hepatocytes as compared to that in the livers of the animals of the remaining groups.

Discussion

Due to the poor survival of rats in the DMN-CCl₄ treated group statistical evaluation was not possible. However, it would seem reasonable to argue that the administration of carbon tetrachloride invoked a regenerative wave of DNA synthesis which would have been superimposed upon the wave induced by DMN.

The antimitotic effect of DMN was responsible for the high level of nuclear polyploidy seen in Group I (DMN + CCl₄) and Group II (DMN). The level of polyploidy in Group I (54%) was considerably higher than that seen in Group II (40%), presumably due to the DNA synthesis induced

by carbon tetrachloride. One must conclude from this that the level of polyploidy induced by DMN during its antimitotic activity is a function of the number of cells stimulated to enter DNA synthesis.

Histological examination of the livers from rats administered both DMN and carbon tetrachloride revealed that by the fourth day of the experiment healing was not as complete as in the livers of rats administered carbon tetrachloride alone. This would no doubt have been due to both the severe degenerative effect induced by the two toxins administered at the same time and the antimitotic effect of DMN, so that the healing time was prolonged.

Experiment 11.2

The mitotic counts following DMN administration to the neonate rat as set out in Chapter 8, showed a significant depression for the first two days only. Inhibition was present or at least appeared to persist until the third day but the level of mitosis was not statistically different from controls at this time. The discussion on this finding suggested that increased numbers of rats in treated and control groups might have given a more definitive result on the antimitotic effect of DMN three days after dosing.

An alternative system to confirm that the effect of DMN persisted for the first three days was devised. This involved provoking a wave of mitosis in the liver on days three and four following the injection of DMN. Such activity should result in increased ploidy levels when

compared to appropriate controls if an antimitotic effect is still present.

Once again carbon tetrachloride was used in order to induce a wave of mitosis in the liver.

Method

Two-day old rats were injected intraperitoneally according to the schedule shown below. All injections were performed at 10 a.m. on the appropriate days.

Group I	Four rats.	10 mg DMN/kg body weight followed three days later with 0.1 mg CCl ₄ /kg body weight
Group II	Four rats.	10 mg DMN/kg body weight followed three days later with 20 µl physiological saline.
Group III	Three rats.	20 µl physiological saline followed three days later with 0.1 mg CCl ₄ /kg body weight.

Three days after the final injection the rats were killed and liver smears prepared, stained with Feulgen aqueous light green and individual nuclei evaluated with regard to their respective DNA contents. Portions of liver were also fixed in 10% neutral buffered formalin and eventually routinely processed and embedded in paraffin wax. Sections, 4 µ thick, were stained with Ehrlich's alum haematoxylin and eosin.

Results

All the rats survived their respective treatments and the hepatocyte nuclear ploidy populations observed are set out below in Table 11.2.

Table 11.2

Percentage of hepatocyte nuclei in each ploidy class (Ash-Wistar rats given initial dose at 2 days of age, second dose when 5 days old and killed when 8 days old.)

Dosage regimen	Group I			Group II			Group III		
	10 mg DMN/kg body weight			10 mg DMN/kg body weight			20 µl saline		
	0.1 mg CCl ₄ /kg body wt.			20 µl saline			0.1 mg CCl ₄ /kg body weight		
% Nuclei per ploidy class	% 2N	% 4N	% 8N	% 2N	% 4N	% 8N	% 2N	% 4N	% 8N
Individual	52	44	2	67	33	0	95	5	0
rat values	54	43	3	59	39	2	95	5	0
	50	45	5	61	39	0	92	8	0
	54	43	3	66	30	4	-	-	-
Mean values	53	43.75	3.25	63.25	35.25	1.5	94.0	6.0	0

A Student's 't' test carried out on the respective hyperdiploid nuclear proportions of Groups I and II revealed a significant difference $t = 15.6$ with 6 degree of freedom $p < 0.001$.

Histological appraisal of the livers in this experiment was largely as expected. Healing of the lesions induced by carbon tetrachloride in Group III had taken place by the third day following administration. The livers from animals which were given DMN and then physiological saline (Group II) showed the expected variation in nuclear size. The livers of the animals in Group I (DMN followed by CCl_4) showed yet more marked variation in the nuclear size of hepatocytes when compared with the livers of the animals in Group II but were essentially similar in appearance in all other respects.

Discussion

The dose of carbon tetrachloride three days after the injection of DMN was, as shown in previous experiments, administered at a necrotoxic level. This dose thus induced a wave of mitosis and DNA synthesis over at least the next 48 hours. The finding that this group of rats showed increased numbers of hyperdiploid nuclei over those of the animals given the single injection of DMN indicates very strongly that the antimitotic activity of the compound persists in some nuclei for at least four days. The possibility does exist that the tetraploid nuclei in the livers of the animals of Group I which were induced by the first injection of DMN, were preferentially stimulated into mitosis following the necrotoxic action of carbon tetrachloride. However since the experiments described in Chapter 8 gave no indication of preferential mitosis of these

tetraploid nuclei in the normal growth of liver from six days of age onwards this latter explanation is the more unlikely.

It has been established thus far that a single injection of DMN increases cell and nuclear size by mitotic inhibition of one cell cycle. The experiment described below investigates the effect of more prolonged administration of the compound on nuclear ploidy.

Experiment 11.3 - An investigation of the effect of repeated injections of dimethylnitrosamine on the nuclear ploidy of the hepatocytes

Method

A 2-day old litter of six rats was divided so that four animals were dosed with 10 mg DMN/kg body weight and two rats with 20 μ l physiological saline by intraperitoneal injection. This procedure was repeated at four-day intervals so that each animal received in all three injections of appropriate solution. The animals were killed four days following the final injection. Feulgen aqueous light green stained smears of liver were prepared and appraised with regard to the respective ploidy populations of the nuclei of the parenchymal cells. Portions of liver were also fixed in 10% neutral buffered formalin and subsequently routinely processed for histology. Sections were stained with Ehrlich's alum haematoxylin and eosin, Van Geison for collagen and by the method of James for reticulin.

Results

Of the four animals which received three injections of DMN only two survived. The remaining two animals were cannibalised on the sixth and seventh day of the experiment respectively.

No abnormalities were found either macroscopically or microscopically in the tissues of animals in the control group.

The survivors of the treated group were considerably emaciated. Gross examination of the livers revealed a pale mottled appearance and they had a firm consistency. The livers were considerably smaller than those of the control animals and adhesions were present between the surface and the visceral organs and diaphragm. The respective liver weights were as under:-

Treated liver weights	Control liver weights
0.53 g	0.84 g
0.49 g	0.88 g

Microscopically, the livers of the animals which had received three doses of DMN were grossly abnormal in appearance. Areas of necrosis of hepatocytes could be identified centrilobularly which had evoked a minimal inflammatory cell response. These necrotic areas appeared to have coalesced in some regions and in one instance virtual destruction of a complete lobe was observed. Other foci distributed irregularly throughout the liver showed more chronic changes with central zones of necrosis surrounded by fibrosis and bile duct proliferation (Fig. 11.1). The reticulin framework of the liver was grossly distorted by the treatment (Fig. 11.2). There was marked variation in the size of

the nuclei of the parenchymal cells and in many the uptake of haematoxylin was indifferent (Fig. 11.3). The cytoplasm was of a pink granular nature with the occasional large vacuole. Mitotic figures in hepatocytes were by no means numerous but occasional large parenchymal cells could be seen in division (Fig. 11.4). The nuclear ploidy populations of the hepatocytes from these livers are shown in Table 11.3 and are illustrated in histogram form in Text-Fig. 11.2.

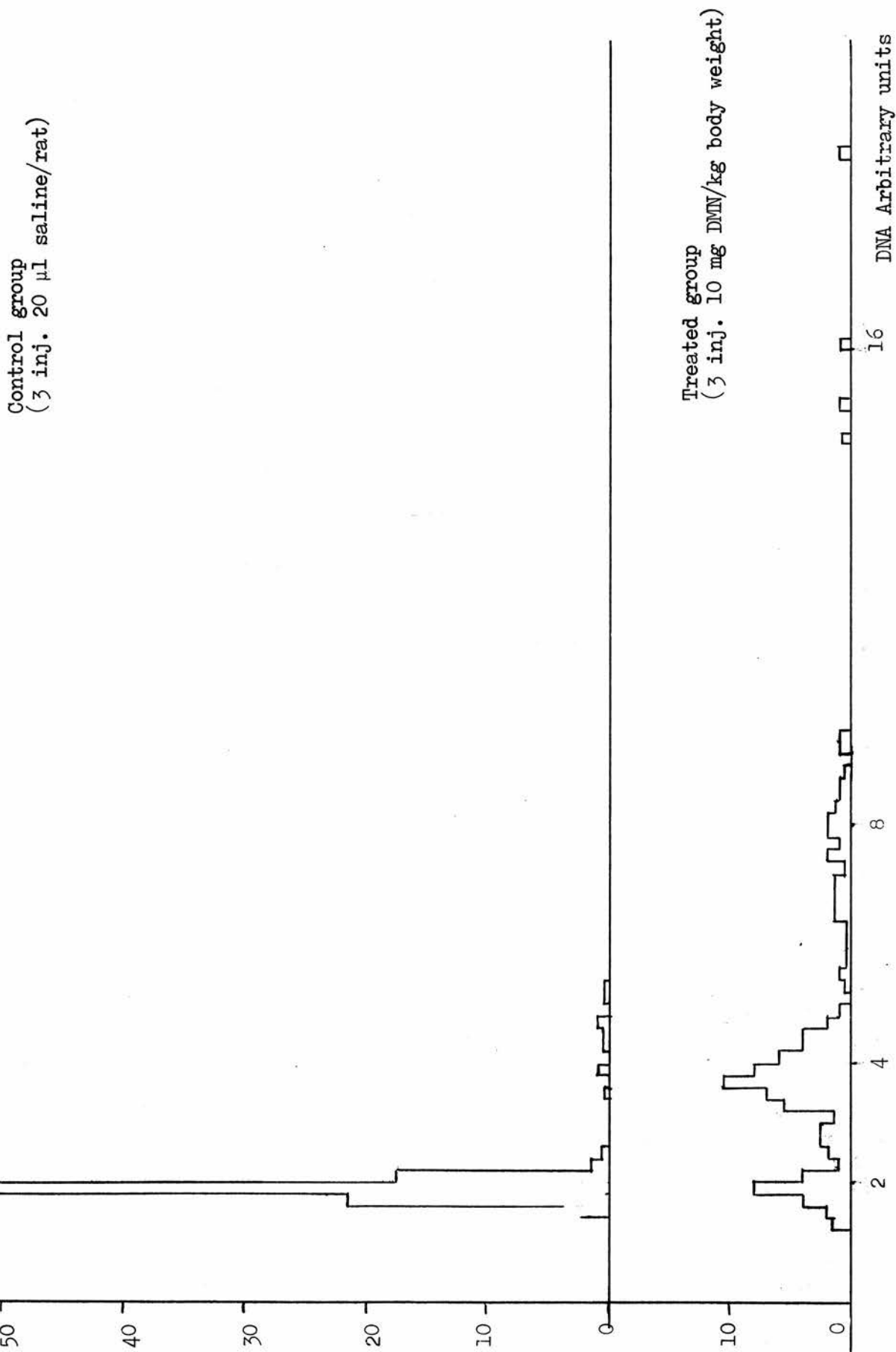
Table 11.3

Percentages of the hepatocyte nuclei in each ploidy class. (Ash-Wistar rats dosed at 2, 6 and 10 days of age; the animals were killed at 14 days of age)

Dosage regimen	Rat No.	Sex	% 2N	% 4N	% 8N	% 16N
3 doses 20 μ l saline i/p	1	M	95	5	0	0
	2	F	97	3	0	0
	Mean		96	4	0	0
3 doses 10 mg DMN/kg body wt. i/p	1	M	23	54	22	1
	2	M	26	49	22	3
	Mean		24.5	51.5	22.0	2.0

Text-Fig. 11.2

Nuclear ploidy in livers from both control rats and rats treated with 10 mg DMN/kg body weight at two, six and ten days of age. The animals were killed four days following final injection.



Discussion

The dosing regimen of 3 injections of DMN, each at 10 mg/kg body weight and spaced at four day intervals resulted in a marked distortion of liver architecture. This effect would have undoubtedly been due to the repeated necrotoxic action of DMN at this level and the subsequent antimitotic activity. This latter facet of the nitrosamine action would have prevented adequate regeneration of liver cells following each injection. The quantitative evaluation of the antimitotic effect is reflected in the marked nuclear ploidy shift with the formation of large numbers of tetraploid and octoploid nuclei and even some with 16N values. On this evidence one must conclude that prolonged administration of DMN leads to hepatocytes being blocked in G₂ of the cell cycle on successive attempts at division. It would seem that the high ploidy nuclei are capable of further division, however, since relatively large parenchymal cells were observed in apparently normal division.

Comparison must once more be made with the corresponding action of the pyrrolizidine alkaloid lasiocarpine. With this compound single dosage leads to continued megalocytosis over many months (Schoental and Magee, 1957; Nolan et al. 1966) and this has been shown to be due to its persistent antimitotic effect (Downing and Peterson, 1968; Jago, 1969; Culvenor et al. 1969). The megalocytes induced by lasiocarpine were observed to be capable of division (Schoental and Magee, 1959) although in contrast to the observations made in the above experiment with DMN, many of the mitotic figures were reported to be abnormal in appearance.

Even prolonged administration of DMN at low levels (Christie and LePage, 1961) results in only moderate cell and nuclear enlargement over a period of months. This is no doubt a reflection of the non-persistent antimitotic effect of DMN on individual hepatocyte nuclei.

ILLUSTRATIONS

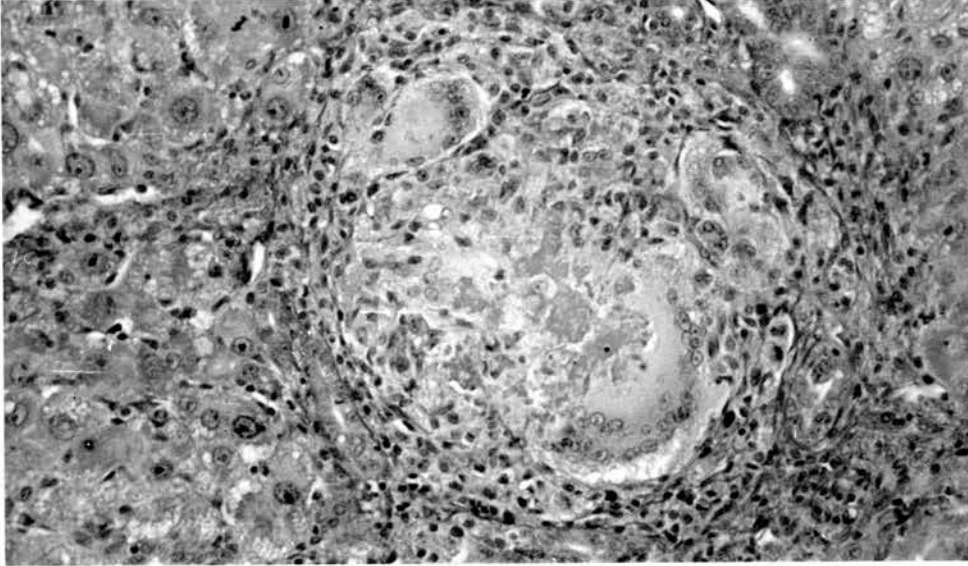


Fig. 11.1 Necrosis, fibrosis and bile duct proliferation in the liver of a rat treated with three injections of DMN each at 10 mg/kg body weight and spaced four days apart (H+E x 360).

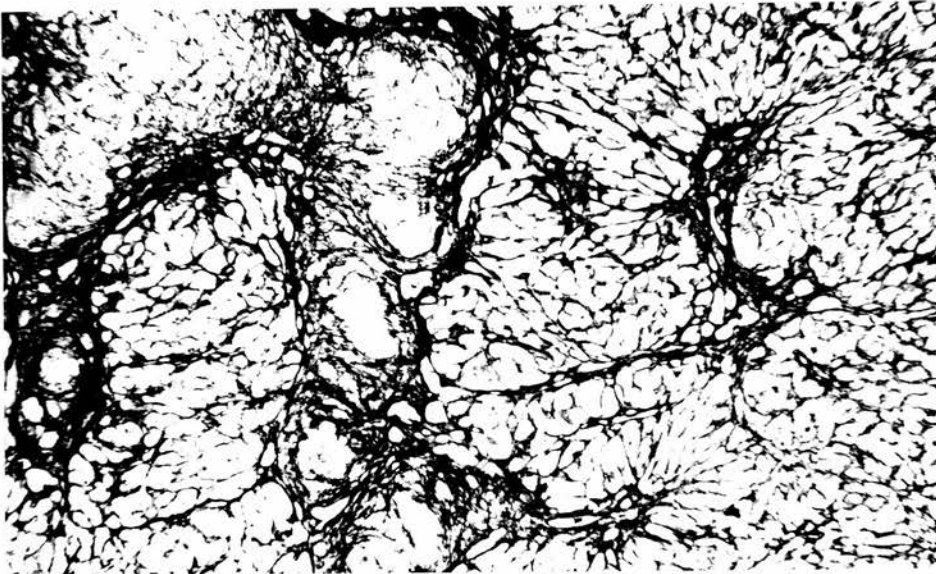


Fig. 11.2 Liver of rat which had received 3 injections of DMN spaced apart at 4-day intervals, showing gross distortion of liver architecture (James' reticulin x 90).

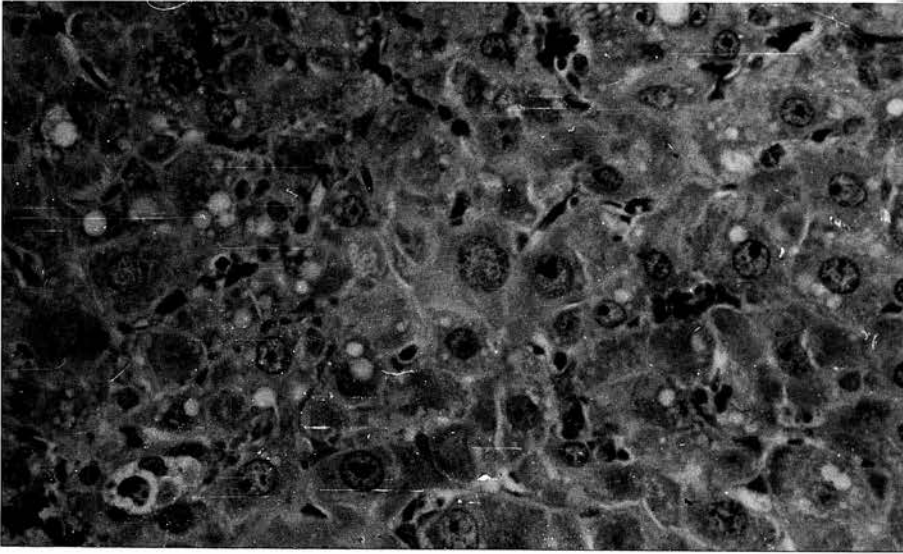


Fig. 11.3 Liver from rat treated as outlined in the legend of Fig. 11.2 above and illustrating the gross variation in nuclear size (H+E x 360).

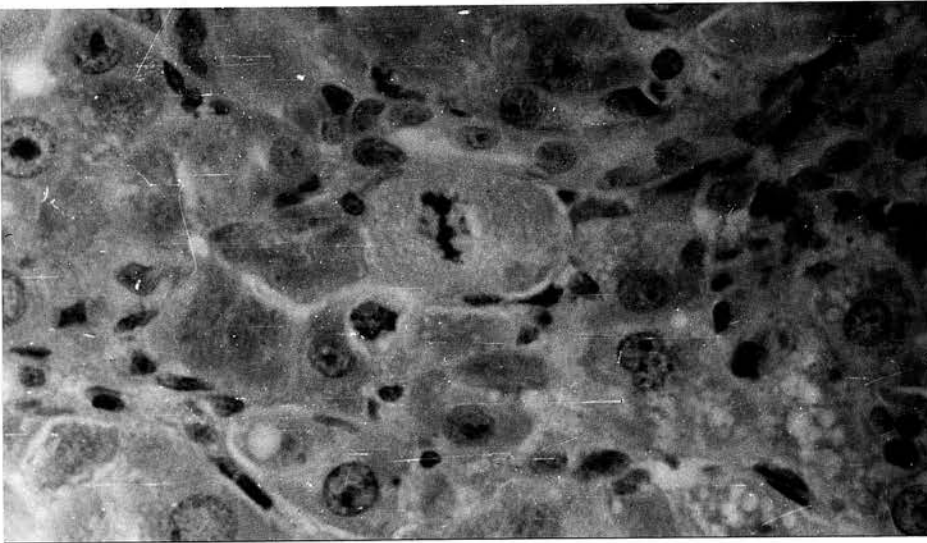


Fig. 11.4 Liver from rat treated as above showing a metaphase mitotic figure in an enlarged hepatocyte (H+E x 550).

CHAPTER 12

GENERATION TIMES OF HYPERDIPLOID HEPATOCYTES

INDUCED BY DIMETHYLNITROSAMINE

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The immediate action of dimethylnitrosamine on the cell cycle of hepatocytes has been established. The experiment described in this chapter was set up to investigate the fate of hepatocytes involved in the ploidy increase evoked by this acute action of DMN. Using an autoradiographic technique, the generation times of parenchymal cells labelled over the short time during which DMN was exerting its anti-mitotic effect was compared with the generation times of hepatocytes in appropriate control livers, over a period of six months. (Generation time in this context is taken to mean the time between the formation of a cell by mitosis and the time when this cell itself undergoes mitosis).

In order to produce a high labelling index in the hepatocyte population two pulse-labels of tritiated thymidine were given six-hours apart. These injections were given twenty-four and thirty hours respectively following the administration of DMN. The duration of DNA synthesis of hepatocytes over the first week of life has been given as seven hours (Post and Hoffman, 1964) and the above system was thus thought to ensure that the majority of hepatocytes labelled during the first pulse would be unlikely to be labelled again by the second thymidine administration. Moreover, the administration of the radioactive label at twenty-four and thirty-hours following DMN dosage ensured that the wave of DNA synthesis evoked by this compound was utilised to the full to produce a high labelling index.

Post and Hoffman (1961; 1967) have shown that dosage levels of tritiated thymidine of 2 $\mu\text{c/gm}$ body weight and above can in itself lead

to increased ploidy levels in the young rat, possibly through a mechanism involving mitotic inhibition. Appropriate controls were therefore included to validate the dosing regimen of tritiated thymidine in this experiment in order to demonstrate that it had no effect on the nuclear ploidy of the liver.

Method

Litters of two-day old rats were divided into two groups and dosed by intraperitoneal injection with 10 mg DMN/kg body weight or 20 μ l physiological saline at 10 a.m. Twenty-four hours later these animals received an intraperitoneal injection of tritiated thymidine at a dose level of 1 μ c/gm body weight. This injection was repeated at thirty hours.

The protocol for killing the animals over the six month period is set out in Table 12.1.

Table 12.1

The protocol of times following DMN administration
when animals were killed

Time of death following DMN or physiological saline	No. of animals in control group	No. of animals in treated group	
31 hours	2 M	1 M	1 F
3 weeks	2 M 1 F	2 M	1 F
6 weeks	2 M 2 F	2 M	2 F
12 weeks	1 M 2 F	3 M	2 F
24 weeks	2 M 3 F	3 M	2 F

M = Male

F = Female

When the animals had been killed, the livers were processed for autoradiography as has already been described (p 119). The thirty-one hour, three-week and six-week liver samples were sectioned and exposed together. The twelve-week and twenty-four week liver samples were sectioned and exposed simultaneously but a positive control section from the thirty-one hour group was included so that valid comparisons between these two exposure groups could be made with regard to respective grain counts. All exposures were of exactly three weeks.

Sections were appraised by scoring the number of hepatocyte nuclei labelled (≥ 5 grains was accepted as a positive label) and with respect to the degree of labelling (grain count; number of grains overlying any positively labelled nucleus).

With the thirty-one hour group, one thousand hepatocyte nuclei were scored in each liver sample using the x 100 oil emmersion objective lens. All the nuclei in a field were examined and results recorded before a new field was selected. With each of the three, six and twelve-week liver sections, three thousand hepatocyte nuclei were scored. The numbers of labelled hepatocyte nuclei in the livers of the animals sacrificed after twenty-four weeks were so sparse that a different counting procedure was adopted for this group: first, the liver sections from each sample were systematically scanned under the x 40 objective lens; the number of fields scanned and the number and degree of hepatocyte nuclei labelled were recorded. Then the total number of hepatocyte nuclei in ten fields was counted and from this, a mean nuclear number per field estimated. The approximate labelling index could then be

calculated from the number of nuclei observed as labelled, the actual number of fields scanned and the mean nuclear number/field.

An additional control procedure was carried out to ensure that the level of tritiated thymidine used in the above experiment did not in itself interfere with the cell cycle of the hepatocytes. Portions of liver from the three rats in the control group killed at the three week interval were disaggregated with TPB solution and the nuclear ploidy population of the livers appraised by Feulgen microspectrophotometry, in order that any increase in nuclear ploidy through mitotic inhibition could be detected.

Autoradiographs were also prepared from the kidneys of the animals in this experiment for subjective appraisal of cell labelling at the various time intervals.

Duplicate sections of liver and kidney from each animal were routinely stained with Ehrlich's alum haematoxylin and eosin as the histological morphology of the methyl-green pyronin stained autoradiographs was found to be difficult to interpret. Appropriate sections were also stained by Van Geison's or by the combined Alcian Blue-Periodic Acid Schiff method.

Results

1. Liver

The microspectrophotometric examination showed that the proportion of tetraploid nuclei in the three control rat livers killed three weeks after the administration of tritiated thymidine did not exceed 6%. It was concluded from this result that the level of radioactivity

administered ~~did~~not in itself interfere to any significant degree with the cell cycle of hepatocytes. Had it done so an increase in the proportion of tetraploid nuclei would have resulted, above the level found in the control nuclear ploidy values seen in Experiment 8.1.

The degree of labelling of the parenchymal nuclei of the liver autoradiograph exposed with the livers of the animals of the first three time intervals and on the second occasion as a positive control with the twelve and twenty-four week studies was as follows:-

	<u>Exposure 1</u>	<u>Exposure 2</u> (control)
Percentage labelled nuclei	6.5	6.1
Mean grain count/nucleus	23.4 \pm 12.6	20.4 \pm 10.8

Both the percentage labelled nuclei and the mean nuclear grain counts are almost identical in the two exposure groups. A Student's 't' test carried out on the means of the grain counts revealed no significant difference $p > 0.1$. On this evidence it was concluded that valid comparisons could be made between the autoradiographs exposed on the two separate occasions.

a) Autoradiography

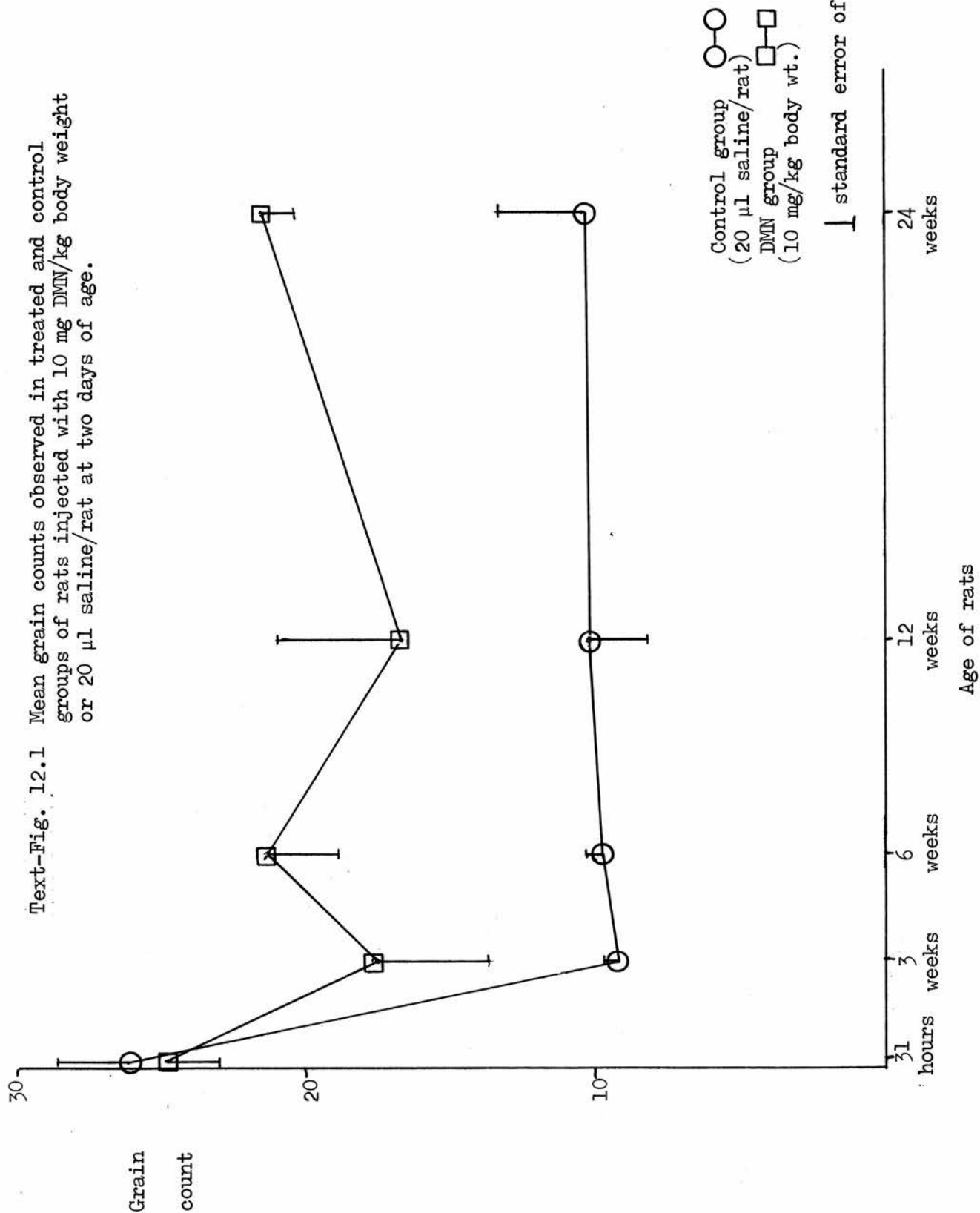
Table 12.2 shows the mean percentage of labelled cells in the treated and control groups at each of the time intervals studied. Text-Fig. 12.1 illustrates graphically the mean grain counts of each group at the various time intervals.

Table 12.2

Mean percentage labelled cells in DMN and control groups at varying intervals following injection at two days of age (p calculated by Student's t test at each time interval)

Time following administration DMN	Percentage labelled cells \pm SD		p level of sig. of difference between means
	Control	Treated	
30 hours	6.8 \pm 0.98	28.2 \pm 2.97	<0.02
3 weeks	0.5 \pm 0.17	0.84 \pm 0.22	<0.01
6 weeks	0.41 \pm 0.11	0.46 \pm 0.21	Not significant
12 weeks	0.37 \pm 0.07	0.32 \pm 0.20	" "
24 weeks	0.0093 \pm 0.0045	0.0543 \pm 0.028	<0.001

Text-Fig. 12.1 Mean grain counts observed in treated and control groups of rats injected with 10 mg DMN/kg body weight or 20 μ l saline/rat at two days of age.



There was no obvious lobar or lobular pattern to the distribution of the labelled hepatocyte nuclei in the autoradiographs of livers from the treated and control rats at the 31 hour time interval. After this time, however, the labelled hepatocyte nuclei showed a definite preferential location in periportal areas (Fig. 12.1). Other cell types such as bile duct epithelium and Kupffer cells were also labelled by the tritiated thymidine, but the subjective opinion was formed that the degree of labelling of these cellular constituents was the same in both treated and control groups throughout the period studied.

b) Post mortem findings

Abnormalities were noted in the liver and kidneys on macroscopic examination.

The macroscopic appearance of the livers of the rats treated with DMN and killed 31 hours later was one of pale mottling when compared to the appropriate controls. Microscopic examination of these livers revealed the irregular patchy necrosis of hepatocytes which has already been described in Chapter 6. The livers of the rats treated with DMN and killed at the remaining time intervals up to three months revealed no abnormalities. A number of liver lesions were noted in rats killed at six months however. One male and two female rats killed at this time showed macroscopic lesions in the liver. In each of the livers of two of these animals was a single slightly raised nodule, 2-3 mm in diameter which on cutting appeared white and cellular. Histological examination showed these lesions to be small foci of hepatocytes, the growth of which had slightly compressed the liver parenchyma (Fig. 12.2). As outlined in the following chapter it was decided to describe this type of

change as that of nodular hyperplasia. The other rat had a liver which exhibited two macroscopic lesions. One of these foci was approximately 0.5 cm in diameter and was considerably raised above the surface of the liver. On the cut surface it had a somewhat haemorrhagic appearance and microscopically it was seen as a small hepatoma with large cavernous blood sinuses (Fig. 12.3). The second lesion seen in the liver of this animal proved to be a small area of nodular hyperplasia. This liver also showed a number of small foci (which had not been obvious macroscopically) consisting of hepatocytes with grossly vacuolated cytoplasm. In the experiments of the next chapter these lesions of vacuolated cells were shown to be areas of hepatocytes undergoing fatty change and were seen in both control and treated animals.

These lesions did not differ in nature to those observed in a carcinogenic study described in the next chapter when their nature and significance will be discussed.

2. Kidney

a) Autoradiography

Subjective evaluation of the uptake of tritiated thymidine by the cellular constituents of the kidneys of the treated and control animals in this experiment failed to reveal any significant difference between the two groups.

At the thirty-one hour time interval studied the area of the kidney undergoing the most rapid growth was reflected by the very high level of cell labelling in the zone of the developing nephrogenic cap. The adjacent inner region of the cortex showed fairly numerous labelled

epithelial cells in the tufts of the glomeruli, of the cells in the region of the macula densa, and in the epithelial cells of the convoluted tubules. The ascending and descending limbs of the loops of Henle contained moderate numbers of labelled epithelial cells as did the collecting tubules of the medulla. In the cortico-medullary region large numbers of plump spindle-shaped mesenchymal nuclei were also labelled.

At the three week period the heaviest labelling was in the epithelial cells of the medulla of the kidney. In contrast, the labelled epithelial cells in the cortex were sparsely distributed at this time; this was no doubt a reflection of the fact that growth in this latter region over the first three weeks of life is more rapid than elsewhere. Cells in the glomerular tuft and in the region of the macula densa were still heavily labelled at this time in the inner half-segment of the cortex.

The labelled cells in all regions of the kidney became progressively less numerous over the ensuing five months. The specific areas which appeared to retain the highest level of label up to six months of age were occasional glomerular tufts and cells in close association with the macula densa in the inner-half of the cortex.

b) Post mortem findings

Macroscopic examination revealed lesions in the kidneys of three of the animals at the six-month time interval.

<u>Animal No.</u>	<u>Distribution of lesions</u>	
	<u>Left kidney</u>	<u>Right kidney</u>
79/2 Female	1	-
81/3 Male	2	1
81/4 Male	1	2

Macroscopic and microscopic examination of the kidneys of the remaining animals in this treated group failed to reveal any significant abnormalities at any of the time intervals studied.

The lesions in the kidneys reported above varied in size macroscopically from approximately 2 mm-6 mm in diameter and in all but one case were located in the cortico-medullary region. The exception to this was a small 2 mm diameter lesion which protruded into the pelvis of the kidney. All the lesions showed, on cutting, a white cellular surface of firm consistency.

Microscopically, these lesions were very similar in appearance. The outer zone of these tumours was typically composed of tightly packed spindle cells with slender nuclei with scant chromatin. Many mitoses were noted in these cells and they appeared to be spreading along the supporting connective tissue of the neighbouring kidney parenchyma leading to the destruction of most of the tubules (Fig. 12.4). The central zone consisted of varying amounts of mucoid or fibrous connective tissue, was relatively acellular, and contained remnants of tubules which in one tumour were markedly cystic (Figs. 12.5 and 12.6).

Discussion

In addition to the findings on the fate of the polyploid hepatocytes induced by DMN treatment, additional information was gained from the above experimental work with respect to the carcinogenic action of the compound on both the liver and kidney.

The reparative wave of DNA synthesis following DMN administration was confirmed by the above experiment with a marked increase in labelled

hepatocytes in the livers of the treated animals when compared to appropriate controls. The mean nuclear grain count at this thirty-one hour period was similar in both groups of animals.

By three weeks the proportion of labelled hepatocytes had decreased considerably in both the treated and control groups (see Table 12.2). The livers of the treated animals at this time, however, still exhibited a higher proportion of positive nuclei; this was almost certainly a reflection of the wave of DNA synthesis seen shortly after DMN administration in this group. The mean nuclear grain counts had also decreased considerably by this time (see Fig. 12.1) although this decrease was very much less marked in the hepatocyte population of the treated animals. From this drop in the proportion of labelled hepatocytes, which was accompanied by a relatively much less marked decrease in grain count in the treated as compared to control group, it is concluded that the replication pattern of at least some of the hepatocytes in the treated livers was not identical with that in the control livers. The marked decrease of labelled nuclei in the treated livers by the end of the third week of life supports one of the conclusions of an earlier experiment (p 197) which suggested that most of the mononuclear tetraploid cells formed though the DMN treatment were capable of further division. However, the higher mean grain count per nucleus seen in the livers of the treated animals at the end of the three week period indicates that a small proportion of mononuclear tetraploid cells induced by DMN treatment do not take part in the normal replication pattern of the hepatocytes. Experiment 8.1, it will be recalled,

indicated that there was a slight decrease in the number of hyperdiploid nuclei induced by the DMN treatment over this initial three week period. The probable explanation of this decrease is now made clear by the experiment of this chapter, it being due to a small proportion of the cells blocked in G_2 not participating normally in the replication pattern of the hepatocytes.

Over the three to twelve week period the percentage of labelled hepatocytes steadily declined in both groups but at a far slower rate than during the first three weeks of life. Moreover, nuclear grain counts changed little over this period. At first sight these two facts suggest that little cell division has taken place over this time. Such a conclusion is irreconcilable with the fact that very considerable liver growth takes place from three to twelve weeks of age (McKellar, 1949). It should be remembered in this connection, however, that it is during this time that the main development of physiological polyploidy is taking place (Alfert and Geschwind, 1958; Carriere, 1962; Nadal and Zajdela, 1966). Such a process, as set out in the literature survey of this thesis, involves an intermediate binucleate cell system. This results in an increase in nuclear and cell size but not an increase in nuclear number. Thus the increase in liver mass over this period is due to a large extent to increased cell size, and the slow rate of dilution of labelled hepatocytes is therefore not surprising. In both treated and control groups, the mean nuclear grain count does not alter over this time indicating that the labelled hepatocytes are not entering the proliferative pool of the liver. A population of cells

with long generation times was thus already becoming apparent.

Between the twelve and twenty-four week period studied a very marked drop in the proportion of labelled hepatocytes took place in both treated and control groups although the mean nuclear grain count did not alter. These facts would be compatible with individual highly labelled hepatocytes entering the proliferation pool of the liver and passing through rapid successive divisions resulting in a loss of detectable label. The proportion of labelled parenchymal cells in the treated livers was very much greater than in the control livers at twenty-four weeks, however, once more indicating the non-entrance of a greater proportion of labelled hepatocytes into the proliferative pool, in the DMN livers.

In short, this experiment has shown that the proliferation of the hepatocytes from shortly after birth onwards enables one to divide them into two populations. There is, firstly, a population with a short generation time, the members of which have already divided a number of times by three weeks of age. In contrast to this, there is a population of hepatocytes some of which survive and do not divide for up to at least six months from birth. The cells of this latter population appear to enter gradually the proliferative pool of the liver and then undergo rapid successive divisions. The DMN treatment would seem to increase still further the generation time of this long-lived population of hepatocytes.

McDonald (1961) also detected what appeared to be both a short and a long lived population of hepatocytes in the normal liver of the

adult rat. The results of this worker differed somewhat from the results of the above experiment. McDonald found that only 41% of the newly formed cells divided or died within sixty days while the remainder lived six months or longer. The far greater rate of division shown in the experiment of this chapter is of course due to the fact that the experiment was begun in the two-day old animal rather than the adult, and gross increases in liver mass thus occurred over the experimental period.

The concept of two populations of hepatocytes finds additional support in replication studies by other workers. Post and Hoffman (1964; 1965a) investigated the nature of the replicating pattern of hepatocytes of varying ages and showed that the total number of cells engaged in division (proliferating pool) decreased very considerably with age. It was suggested that the decreasing numerical size of this cohort of replicating cells was through serial activation of progressively smaller groups of cells in the proliferating pool. Once a cell had entered this pool it would then become involved in a number of successive divisions and then once more become mitotically quiescent.

As mentioned above the far greater numbers of labelled cells in the livers of the treated rats at six months of age may be accepted as a reflection of the lesser tendency of the nuclei affected by the DMN treatment to enter this proliferative pool of hepatocytes. This in itself need not be of particular significance since McDonald (1961) found that in adult rats, hepatocytes involved in the restoration of the liver following partial hepatectomy had life-spans up to four times

as long as hepatocytes of control livers. On this evidence it would seem that following chemical or physical damage of the liver a small proportion of hepatocytes remain proliferatively 'dormant' for an undetermined time.

Although the above conclusion is the most likely on current evidence it would nevertheless seem worthwhile at this point to mention recent work on duration of cell cycles in tumours. The long-lived heavily-labelled hepatocytes seen in the experiment of this chapter differed in one respect from those observed by MacDonald (1961) in that they were in many cases, almost certainly blocked in G_2 following DMN administration and would not have divided since that time. Presumably the cells with long generation times observed by MacDonald would have divided at least once as part of the regenerative response to partial hepatectomy. Post and Hoffman (1969) have recently identified a G_2 population of cells in autogenous rodent sarcoma indicating that generation times are considerably longer than those of coexistent replicating normal cells. Similarly Decosse and Gelfant (1968) demonstrated such a population of G_2 cells in transplanted mouse ascites tumour cells. The administration of antilymphocytic serum was reported to have stimulated the progress of these cells into mitosis. Post and Hoffman (1969) stressed the fact, that their own finding, that over 25% of the tumour cells were present as a G_2 population indicated that growth patterns of tumours must be reconsidered in the light of the existence of this cellular compartment. Furthermore these authors suggested that cells in prolonged G_2 may be quiescent for long periods and then resume replication with consequent rapid growth in tumour size.

On a similar basis it would seem reasonable to suggest that the liver cells blocked in G_2 by DMN treatment could after prolonged quiescence become proliferatively active and give rise to populations of tumour cells.

Apart from the above speculation the autoradiographic study gave no indication of any particular hepatocyte population which could be incriminated in the carcinogenic response. This aspect of the action of DMN is discussed more fully in the next chapter.

Similarly, the subjective evaluation of the autoradiographs of the kidneys failed to delineate the cell population which was involved in the carcinogenic response. The histogenesis of kidney tumours has been much discussed. Riopelle and Jasmin (1969) were of the opinion that they arise from undifferentiated interstitial cells in the cortico-medullary region. In the newborn rat this area is represented by a clear zone consisting of spindle cells in an abundant amorphous matrix stretched between capillaries and tubules in parallel structure. When this region in the neonate rat was compared with early tumour tissue arising from what would appear to be the same area six months later the similarities were apparent. Hard and Butler (1970a) from a detailed study of kidney tumours induced by a single administration of DMN to adult rats maintained on a protein-free diet also suggested that the sarcomatoid cells were derived from a single cell type. These authors, however, have produced very strong evidence which suggests that the tumour cells originated from primitive mesenchymal cells in the vicinity of the glomerular hilus and which were possibly of vascular-endothelial

origin (Hard and Butler, 1970b). These conclusions were based on the observations that, following DMN administration, there was increased division of interstitial cells in the immediate vicinity of the glomerular hilus, which was followed in turn by an infiltration of plasma cells into this area. This reaction gradually subsided but around twelve weeks following the injection of DMN, spindle cells with clear nuclei and prominent nucleoli predominated in the region of the glomerular hilus and local invasion then became manifest. The 31 hour autoradiograph in the experiment of this chapter failed to reveal any increase in the number of cells engaged in DNA synthesis in the glomerular hilus in livers of treated animals. Both groups, however, showed marked activity with regard to DNA synthesis of the cells in this region. Histological examination also failed to reveal any infiltration of this area by plasma cells in the three-week old rat livers or proliferation of spindle cells at the later time intervals. Hard and Butler (1970b) stressed the value of fixation of the kidney by perfusion in delineating these juxto-glomerular lesions and it is possible that the routine methods applied in the above experiment were not sufficiently effective in preserving renal architecture to enable one to identify these early changes.

The main conclusions drawn from this experiment were that the dilution of hyperdiploid cells, induced by DMN treatment, over the first three weeks is due to the non-participation of a proportion of these cells in the proliferative pool. This is reflected in the longer generation time of these cells in the livers of the DMN treated animals

although it is concluded that no particular significance can be attached to this with regard to carcinogenic processes. The view that the majority of tetraploid nuclei induced by DMN treatment are able to enter subsequent divisions following arrest in G_2 of the cell cycle has also been further supported by this autoradiographic experiment.

Finally, in the kidney studies, the autoradiographic and histological findings failed to identify the cell type involved in the mesenchymal tumours induced by DMN. The location and appearance of the early lesions which had been induced by six-months suggested that the tumours could have arisen from the undifferentiated interstitial cells of the cortico-medullary zone or as suggested by other workers from the primitive mesenchymal cell in the region of the glomerular hilus in the cortico-medullary zone.

ILLUSTRATIONS

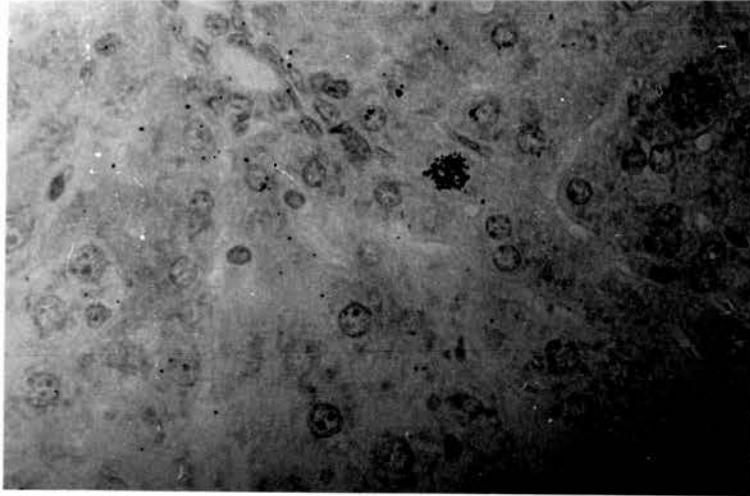


Fig. 12.1 Autoradiograph of the liver of a rat injected six months previously with 10 mg DMN/kg body weight and showing a heavily labelled hepatocyte nucleus in a periportal location (H+E x 550).

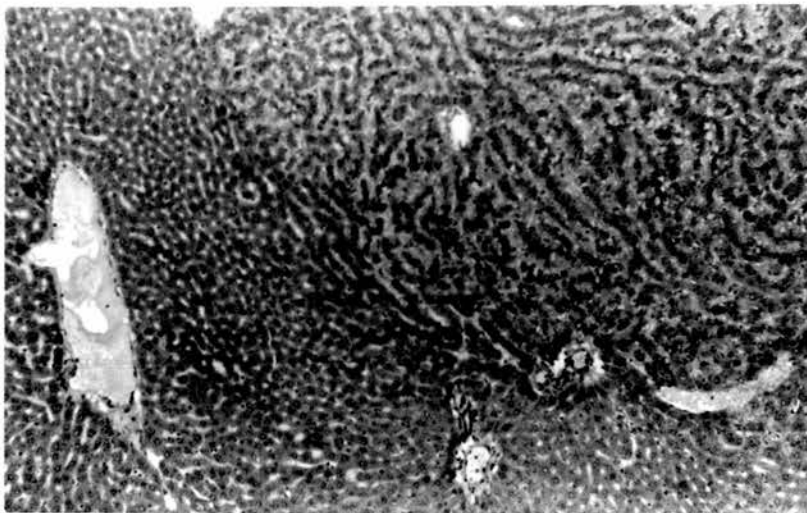


Fig. 12.2 Nodular hyperplasia in liver of a rat treated six months previously with 10 mg DMN/kg body weight (H+E x 90).

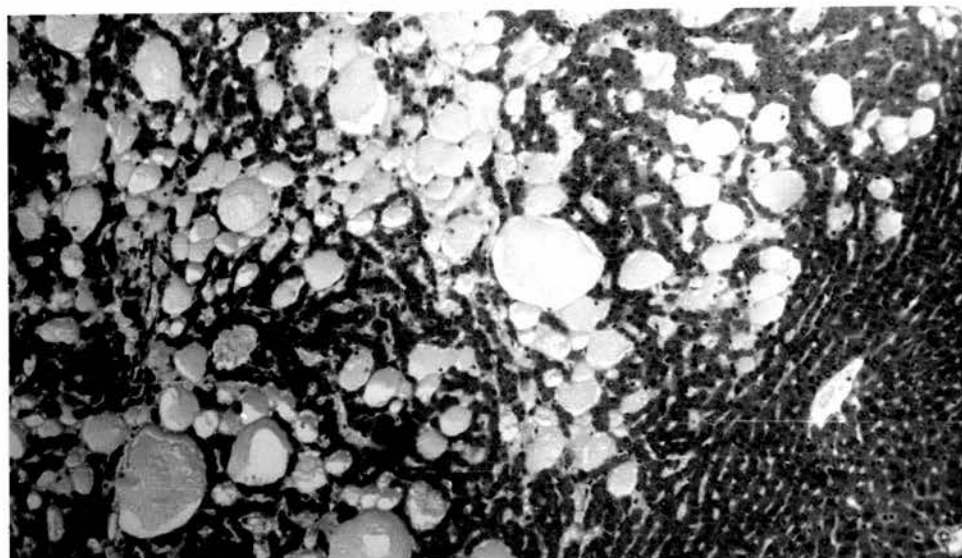


Fig. 12.3 Hepatoma in the liver of a rat dosed six months previously with 10 mg DMN/kg body weight illustrating the large cavernous blood sinuses in the tumour (H+E x 90).

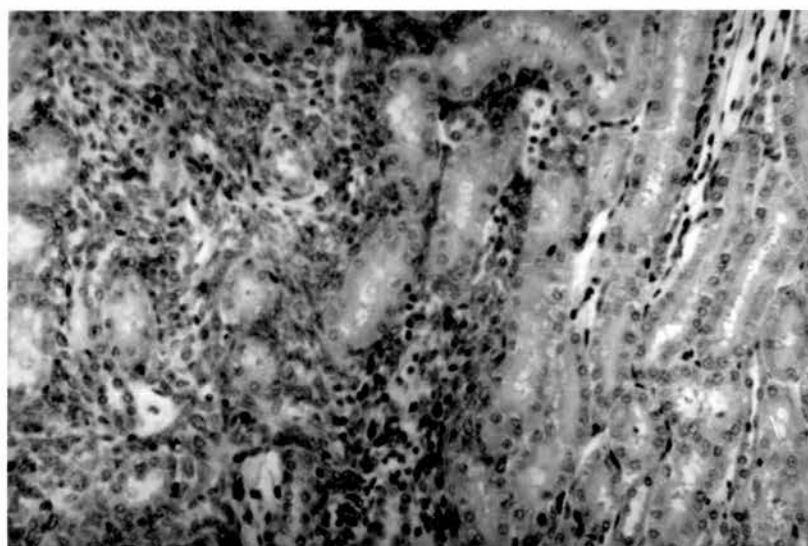


Fig. 12.4 Kidney from a rat dosed with 10 mg DMN/kg body weight six months previously and showing invasion of mesenchymal cells into the neighbouring kidney architecture (H+E x 220).

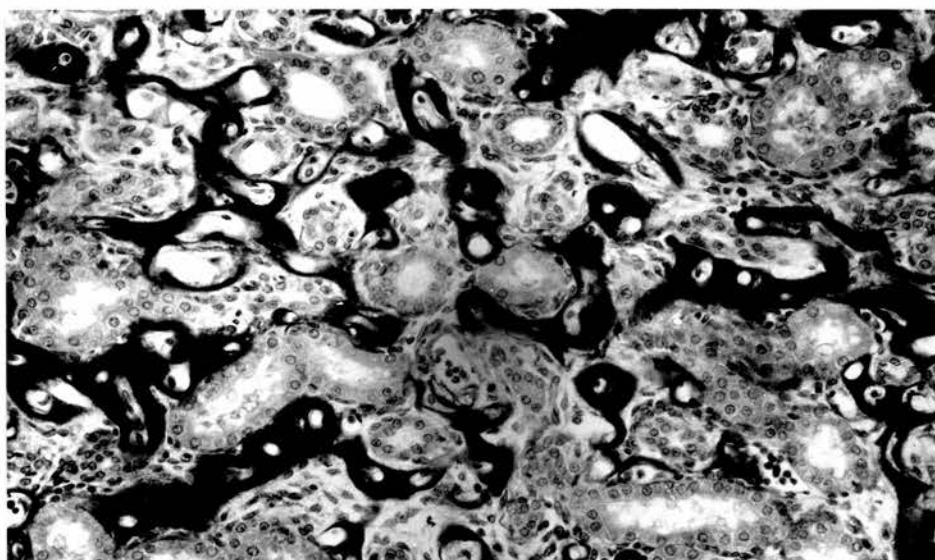


Fig. 12.5 Lesion in the kidney removed from an animal dosed six months previously with 10 mg DMN/kg body weight. This illustrates the central region of a mesenchymal tumour in the cortico-medullary region of the kidney with large amounts of intertubular fibrous connective tissue (Van Geison x 220).

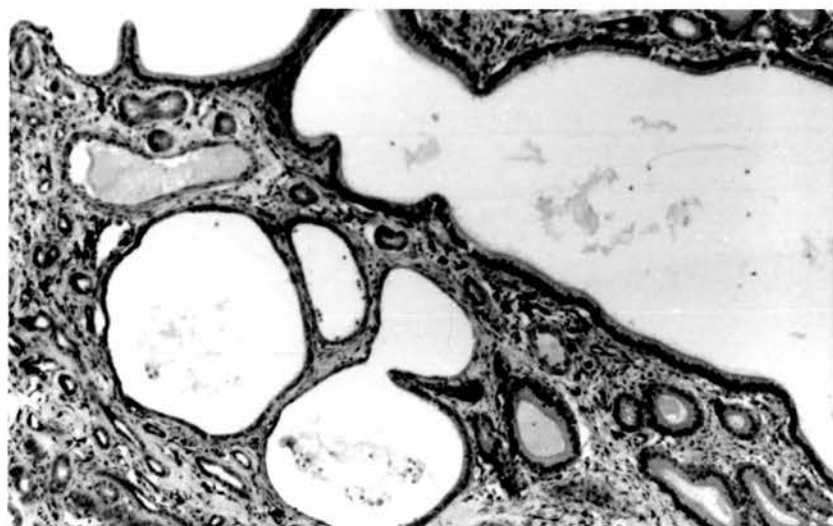


Fig. 12.6 Mesenchymal tumour in the kidney of rat dosed six months previously with 10 mg DMN/kg body weight and showing gross cystic dilatation of tubules (H+E x 90).

CHAPTER 13

CARCINOGENIC STUDIES WITH DIMETHYLNITROSAMINE
AND CARBON TETRACHLORIDE

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The study of cellular kinetics in the liver following administration of DMN and carbon tetrachloride revealed profound differences in the way in which these two compounds induce nuclear enlargement in the liver. These findings were by no means unexpected in the light of the classification in Chapter 3 wherein DMN was tabulated as a potent necrotoxin and carcinogen and carbon tetrachloride as a potent necrotoxin but at most only a weak carcinogen. At the outset of the experimental work it was envisaged that a carcinogenic study with DMN and carbon tetrachloride following single administration to the neonate rat would be necessary to justify such a classification with respect to this particular dosing regimen. The following pages contain a description of this experiment.

The findings of Terracini and Magee (1964) and Terracini (1969) were confirmed in the case of DMN where single administration to the neonate rat induced liver, kidney and lung tumours. A carcinogenic study following single administration of carbon tetrachloride to the neonate rat has not been previously reported; in the following experiment such a dosing regimen failed to induce neoplasia.

Method

Ash-Wistar rats were dosed by intraperitoneal injection at two days of age with 10 mg DMN/kg body weight or 20 μ l physiological saline, or at four days of age with 0.1 ml carbon tetrachloride/kg body weight. These three treatment groups were separated into respective sexes at 21 days of age and housed as nearly as possible five to each cage and allowed free access to pelleted diet (Oxoid pasteurised breeding diet)

and to tap water. When the animals had reached six months of age they were examined weekly for signs of tumours. If a tumour was positively diagnosed by abdominal palpation the animal was housed separately. These individual animals were then allowed to live on as long as possible but were killed by ether euthanasia when the health of the rat was seen to be obviously deteriorating. At 17 months from the commencement of the experiment the remaining animals were killed. Full post mortem examinations were carried out on all animals. Liver, kidney, lung and other selected tissues where appropriate, were fixed in 10% neutral buffered formalin and processed into paraffin blocks. Sections cut at 4 μ were initially stained with Ehrlich's alum haematoxylin and eosin. Selected sections were stained for collagen by the method of Van Geison or by Masson's trichrome technique, for reticulin by the method of James, and by the combined Alcian Blue-Periodic Acid Schiff method for mucopolysaccharides.

Results

The survival of animals over the period of the carcinogenic study is shown in Table 13.1 below.

Table 13.1
Survival of animals up to 17 months of age

Treatment	No. dosed	Weaning 3 weeks	Survival in months						
			11	12	13	14	15	16	17
DMN	28	24 11M 13F	23	18	16	14	- - -	→	14 7M 7F
CCl ₄	31	26 10M 16F	26	26	25	- - -	- - -	→	25 10M 15F
Control	13	12 5M 7F	12	11	- - -	- - -	- - -	→	11 5M 6F

M = Male

F = Female

Post mortem findings

1) Incidental lesions occurring in control, CCl₄
and DMN treated rats

Lesions arising in both control and treated groups, and which were considered to have occurred spontaneously, were seen in the liver, lungs and kidneys.

In the livers of most animals, in all three groups, occasional foci of lymphocytes were observed in the sinusoidal spaces or associated with portal tracts. There were also occasional areas of hepatocytes, up to approximately one half the size of a lobule, which were undergoing fatty change as demonstrated by oil red O staining.

In the lungs small white 1-2 mm slightly elevated foci were seen and these were confined mainly to the dorsal surface of all lobes; on gross examination it was not easy to be certain of the degree to which these lesions extended into the cut surface but microscopically they were seen as small areas, the alveolar spaces of which were packed with foamy macrophages. Such lesions ascribed as lipidosis, Beaver et al. (1963), are normally found in adult rat lungs. Occasional foci of peribronchiolar lymphoid hyperplasia were also seen in both control and treated animals and again this is a frequent finding in rats in which some degree of chronic murine pneumonia is present (Innes et al., 1956).

In the kidneys of both treated and control animals there was a variable background pathology of chronic nephritis with thickening of glomerular basement membranes, dilatation of tubules, protein casts within tubules and interstitial foci of fibrosis and lymphocytes. As will be outlined below animals treated with DMN and which developed

kidney tumours, showed nephritis which was often considerably worse in surviving remnants of the organ when compared to the other kidney or kidneys from control animals.

In the DMN treated group, a mass of tissue was observed in one male rat which was killed in a moribund state 13 months after commencement of the experiment. This tissue mass was attached to the dorsal wall of the abdominal cavity in the pelvic region. When dissected free the lesion appeared to be part of the reproductive tract with the two vas deferentia leading into it. The mass was approximately 2 cm across with a smooth external appearance. After bisection, however, the exposed surface presented a picture of hard white cellular tissue in which were sited large soft areas of white fluid material. Histologically this tissue mass was seen to be a lesion of the prostate with broad bands of connective tissue containing very large mast cells and surrounding hyperplastic glandular foci of epithelial cells undergoing necrosis. There was a massive neutrophil polymorpholeukocyte infiltration of these degenerative areas. On the basis of these observations a hyperplastic prostatitis was diagnosed.

In the liver of a female rat killed at the termination of the experiment was found a 2 mm slightly raised cystic-like lesion on the visceral surface of the right lateral lobe. Histologically the cyst was found to contain a helminth which had no alimentary tract and contained calcareous corpuscles. The lesion was thus diagnosed as the cercocystis larva of the tapeworm Hymenolepis nana, a parasite which is capable of developing without an intermediate host in the rat.

2) Tumours found in control, CCl₄ and DMN treated rats

Apart from one tumour found in a control rat all the tumours found in this experiment arose in animals which had been administered DMN.

A) Control animals

The one tumour observed in the control group was found in a 65 week old female rat and consisted of a large white mass approximately 2½ cm across connected ventrally with an equal sized cystic mass. (Fig. 13.1). The lesion was seemingly part of the reproductive tract in the region of the cervix and vagina. On bisection the solid mass contain an obvious lumen surrounded by tissue which was mainly hard, white and cellular, but interspersed with occasional soft cystic areas. The ventral cystic lesion contained thin clear gelatinous material. Histologically, the tumour surrounded irregular spaces lined by stratified squamous epithelium from three to six cells thick. In some areas the epithelial layer was ulcerated, with neutrophil polymorph infiltration. The tumour mass was composed of bundles of spindle shaped vesicular nuclei in which mitotic figures were only rarely seen. In Masson trichrome stained sections small amounts of red cytoplasm was visible and the cells were separated by moderately numerous blue fibres. In other extensive locations, stellate mesenchymal cells with abundant faintly PAS positive mucinous intercellular substance was seen. The edge of the tumour was represented in some areas by a smooth muscle coat and in others by a connective tissue and fat cell capsule. On the basis of the above observations a vaginal fibroma was diagnosed.

B) Animals dosed with carbon tetrachloride

No tumours were observed in the animals dosed with carbon tetrachloride. The reason for the death of one animal in this group after 13 months was not established due to gross post mortem change in all tissues. The livers and kidneys from all the animals killed after 17 months had a similar appearance both macroscopically and histologically to those of the controls, apart from the tapeworm lesion found in one control animal and described above.

C) Animals treated with DMN

A summary of the findings in this group is shown in Table 13.2.

A large number of tumours arose in the group of rats administered DMN at two days of age. The majority of these tumours occurred in the liver and/or the kidneys although a tumour of the lung was also observed in one animal of this group.

a) Observations on the livers of DMN treated animals

There were a number of pathological changes to be seen in the livers of the animals dosed with DMN.

The livers of all rats showed red depressed areas on the capsular surface (Fig. 13.2) although these lesions varied in extent from one animal to another. On microscopic examination, these foci were telangiectic in nature appearing as areas of slight collapse of the capsular surface. The depressed capsular areas were underlain by liver architecture which varied between grossly dilated sinusoids and actual cavernous blood sinuses.

Transluscent, level lesions measuring about 1 mm in diameter were

Table 13.2

Distribution of tumours in the kidney and lungs and of tumours and hyperplastic nodules in the livers of rats treated with a single injection of 10 mg DMN/kg body weight at 2 days of age

[illegible]

seen on the capsular surface of the livers of six rats dosed with DMN. Bisection of the lesions revealed their cystic nature. The cystic contents were faintly eosinophilic in haemotoxylin-eosin stained sections. Frozen sections stained with oil red O showed that lipid was an important component of these lesions and it was thought that they might have arisen from gross fatty degeneration of hepatocytes which originally constituted these areas.

Small solitary elevated nodules, up to 5 mm across, were seen on the capsular surface of the livers of seven rats. The appearance of one of the larger nodules found may be seen in Fig. 13.3a. The distribution of these nodules in the lobes of the respective animals was four in the left lateral lobe, two in the medial lobe and one in the right lateral lobe. The cut surface of these lesions had a white cellular appearance. Histologically, these nodules were composed of hepatocytes poorly arranged into cords. There was a clear demarcation between the normal liver and the nodule with a narrow zone of compressed hepatocytes without evidence of fibrosis (Fig. 13.4a). The lesions always contained varying numbers of portal tracts and central veins according to the size of the nodule. Usually the hepatocytes varied considerably in size and were often considerably larger than those of the surrounding normal liver parenchyma. The nuclei were usually pale and pleomorphic and often contained three or four large nucleoli. Mitotic figures were seldom seen in these foci. The cytoplasm of the hepatocytes was either of an eosinophilic granular nature or grossly vacuolated. In the cells of nodules in livers from two animals were seen large basophilic inclusion bodies (Fig. 13.4b).

Similar nodules, too small to be recognised on macroscopic examination, were seen microscopically in the livers of six other animals. In the liver of five of these rats they occurred as multiple foci as many as five being identified in one instance. In addition, some areas of enlarged cells and abnormal nuclei were found in a number of rat livers which may have represented early nodular formation but no evidence of compression of normal liver was found surrounding these foci.

Only one lesion, seen macroscopically as a large white elevated mass (Fig. 13.5) with a white cellular but slightly cystic cut surface, was considered to be an hepatocellular adenoma. This tumour, approximately 2 cm across, occupied the majority of the left lateral lobe. Histologically, the margin of the growth was very irregular and was surrounded by a very narrow compression capsule of normal liver cells. The tumour itself had no resemblance to normal liver architecture and consisted mainly of expansive sheets of varying sized hepatocytes with abundant but often vacuolated cytoplasm (Fig. 13.6). The nuclei were often enlarged, pale and pleomorphic and mitotic figures were frequently seen. Towards the centre of the tumour occasional inter-sections of connective tissue were seen surrounding cystic bile ducts. These ducts were lined by tall, well delineated columnar epithelial cells with abundant eosinophilic cytoplasm. In some instances the epithelium had formed hyperplastic proliferations into the ductular lumen. Single cell necrosis was observed frequently throughout the tumour mass and also occasional large confluent areas of cell death surrounded by fibrous tissue.

Bile duct adenomata were seen in the livers of two animals. Macroscopically these were seen as raised nodular semitranslucent areas which on cutting exhibited cysts containing watery gelatinous fluid. The gross appearance of the largest of these tumours is seen illustrated in Fig. 13.3a and was evident throughout most of the sectioned surface of the medial lobe (approximately 2-3 cm across). Histologically, although the edge of the tumour mass was sharply defined there was no fibrous capsule. The growth consisted of groups of irregular cystic spaces lined by up to three layers of cuboidal epithelial cells. These bile duct structures were separated by a delicate connective tissue stroma containing blood capillaries and macrophages with engulfed bile pigment (Fig. 13.3b).

b) Observations on the kidneys of DMN treated animals

There was a diversity in the appearance of the kidney tumours both macroscopically and microscopically although in all cases but one they appeared to be of mesenchymal origin. Broadly speaking, in terms of the neoplastic behaviour of these kidney tumours within the period studied, there were three basic macroscopic patterns of aberrant growth. These were respectively (i) small firm nodular tumours usually approximately 1 cm across; (ii) intermediate sized firm nodular tumours replacing up to two-thirds of the kidney but bringing about only moderate enlargement of the organ and, lastly; (iii) gross tumours which were many times the size of and replaced almost completely the original kidney from which they had arisen. In most cases in this latter group the tumours were soft and haemorrhagic but in two cases were hard

firm growths. As will be seen from the following descriptions all these tumours, although they differed markedly in their gross appearance, nevertheless had many histological features in common.

i) The smallest kidney tumours were very much as has been briefly described in Chapter 12 and were seen in kidneys of five of the treated animals. One such tumour was observed on histological examination only and was seen as small aggregations of spindle cells proliferating between collecting ducts in the corticomedullary junction. The nuclei were pleomorphic (oval to spindle shaped) with scant amounts of chromatin and contained, in the majority of cases, one nucleolus only. Mitotic figures were observed frequently in this zone. The cytoplasm was sparse and slightly basophilic and cell outlines were indistinct. Van Geison staining showed the small amounts of intercellular substance to be collagenous in nature.

The remaining tumours in this group were approximately 1 cm across and were seen in all but one instance as white elevated lesions on the greater curvature. Histologically two of these tumours were very similar and extended through the cortex to the level of the corticomedullary junction. The outer zone was composed of densely packed spindle cells infiltrating the normal renal architecture. Towards the centre of the growth, the tumour was much less cellular and both mucoid and collagenous intercellular substance was identified by PAS and Van Geison staining respectively. Within the outer perimeter of the tumour, remnants of tubules could be seen, often cystic or with hyperplastic epithelium, together with occasional dilated Bowmans capsules containing surprisingly intact capillary tufts, (Fig. 13.7).

Another tumour of this series was also situated in the cortex and had a white, translucent slightly cystic cut surface. At the cortico-medullary junction were the usual infiltrating densely packed spindle shaped mesenchymal cells. Above this area, in the cortex of the organ, there were large cystic spaces (Fig. 13.8) containing small shrunken colloidal deposits. Finally, just under the capsule there was a small zone of compressed more normal looking tissue containing tubules and glomeruli. The cysts in the lesion were lined by a single layer of cuboidal to flattened epithelial cells in which no mitoses were noted. It was concluded that these cysts were not in themselves part of a neoplastic process but may have resulted from interference with the patency of nephrons by the mesenchymal growth in the cortico-medullary region.

The final tumour of this series had grown in the form of a polypoid mass about 1 cm in diameter out of the pelvic region of the kidney (Fig. 13.9). The tumour cells in this instance were widely spaced and often stellate in shape. The intercellular substance was faintly PAS-positive and therefore myxoid in nature. This tumour also contained numerous large cystic spaces lined by flattened epithelial cells and was contained within a thin fibrous capsule.

ii) The intermediate sized tumours, seen in three animals, appeared very much as the small nodular growths described above but occupied up to two-thirds of the kidney (Fig. 13.10). Again the peripheral invasive zone was of mesenchymal cells with pleomorphic lightly staining spindle shaped nuclei. More centrally the tumour was much less cellular. Stellate mesenchymal cells were once more associated with a faintly PAS-

positive mucoid intercellular substance and there were fibroblast-like spindle shaped cells associated with collagen formation. The impression was gained that much of the larger fibrous areas were composed of mature granulation tissue indicating that it was part of a repair process following on areas of tumour cell degeneration, or degeneration of normal kidney tissue brought about by adjacent tumour growth. Remnants of tubules, as in the smaller mesenchymal growths, varied in appearance from the grossly cystic, lined with flattened epithelium, to those which were hyperplastic and contained in some instances no lumina at all. Often these tubular remnants were surrounded by fairly well developed smooth muscle fibres as identified by Masson trichrome staining (Fig. 13.11). In one of the kidneys containing this type of tumour was seen a small adenoma consisting of large epithelial cells with abundant eosinophilic cytoplasm and small darkly stained nuclei (Fig. 13.12). This was the only example of adenomatous growth found in the series. Generally, within these intermediate sized tumours, the areas underlying the transitional epithelium of the pelvis (which was always intact although often somewhat distorted) were very much more cellular and contained whorls of mesenchymal cells surrounding isolated collecting ducts (Fig. 13.13).

iii) The largest tumours which almost entirely replaced the kidney were of two main types.

a) One series presented a hard white enlarged lesion up to three times the size of the normal kidney (Fig. 13.14 left kidney). Histologically a small remnant of relatively normal kidney was found

compressed to one side of the growth. These areas showed advanced chronic interstitial nephritis immediately adjacent to the tumour with extreme interstitial fibrosis. Microscopically fibrogenic and mucogenic areas were again identified and Masson trichrome staining demonstrated large areas of smooth muscle fibres within a well developed reticulin framework. Aberrant remnants of isolated kidney tubules were once more observed within the tumour mass.

The unaffected kidney of one of these animals showed marked nephrocalcinosis (as identified by chloranilic acid staining) in the outer cortex, in what appeared to be proximal convoluted tubules.

b) The largest type of tumour observed in the kidneys of six rats were gross enough to considerably distend the abdomen (Fig. 13.14 right kidney and Fig. 13.15). The sudden deaths seen in this experiment were usually due to rupture of the kidney capsule overlying this type of tumour with subsequent fatal haemorrhage into the peritoneal cavity. These growths had a lobulated external appearance and a markedly vascular cut surface of mostly soft consistency. As expected from their macroscopic appearance these tumours varied markedly from one area of the neoplasm to another although they had many features in common with the smaller tumours described above. Usually a remnant of kidney was found although as with the two smaller tumours described above it always showed marked nephritis with gross interstitial fibrosis. Immediately adjacent to this remnant, tumour cells were seen to be invading the renal architecture. The infiltrative cells were again densely packed with ovoid to spindle shaped nuclei with fragmented and scanty chromatin and sparse basophilic cytoplasm. Once more the growth

of these cells was actively destroying renal tubules in the infiltrative zone. Further into the tumour only cystic or hyperplastic remnants of tubules and glomeruli remained which were surrounded by mucoid connective tissue or concentric layers of mesenchymal tumour cells of either fibroblastic or smooth muscle type. A very characteristic feature of these tumours was their very vascular nature with large cavernous sinuses interspersed in the growth and occasional large areas of haemorrhagic necrosis. Towards the central zone of the tumour were large areas of fibrosis which mingled almost imperceptibly into more obviously neoplastic regions. Once again it was felt that these areas of marked collagenic deposition could have represented a replacement of previously necrotic tumour tissue by granulation tissue. In no instance did these tumours appear to penetrate the transitional epithelium of the pelvis although anaplastic tumour cells were thickly packed just beneath the basement membrane. Slightly further into the tumour what appeared to be remnants of transitional or collective duct epithelium were entrapped by the advancing tumour extension and had become hyperplastic.

c) Other tumours found in DMN treated rats

A small adenoma in the lung was noted incidently on histological examination in one animal which died one year after the commencement of the experiment. This rat also had liver and kidney tumours.

Discussion

The above results clearly show that under the particular dosing regimen of this experiment DMN is a potent carcinogen whilst carbon

tetrachloride shows no activity in this respect. DMN induced tumours in the liver and kidney and possibly was also responsible for the lung adenoma seen.

Only one tumour was noted in the control group of rats while none at all were observed in those animals administered carbon tetrachloride.

The finding of a high incidence of neoplasia in the DMN treated group affords an opportunity for discussion of the nature, histogenesis and mechanism of induction of the neoplasms observed.

Small nodules of hepatocytes were noted macroscopically in the livers of seven rats and incidentally on microscopical examination of the livers of six other rats. These lesions, although producing a compression capsule and having no regular cord-like arrangement, contained central veins and portal tracts. The size of the hepatocytes varied markedly in size but mitotic figures were rare. It was concluded that it would not be justifiable to classify these foci as early hepatomata but as areas of nodular hyperplasia only. Whether or not they would have eventually progressed to overt neoplasia must remain an open question. The only unequivocal hepatocellular tumour was the adenoma found in the liver of one rat and this showed many mitoses and bizarre organization and orientation of cells.

Terracini and Magee (1964) also found a low incidence of liver tumours after 53 weeks (1 out of 29 developed a hepatoma in Wistar/Porton rats which survived to weaning age having been injected subcutaneously with 125 μ g DMN within the first 24 hours of life). The histology of the lesions was not described in this publication. In marked contrast Terracini (1969) found an incidence of approximately

60 percent liver tumours in Wistar/Porton rats administered 125 µg subcutaneously within 24 hours of birth. These tumours were described as trabecular or anaplastic hepatocellular carcinomas sometimes with lung metastases. The increased incidence seen in the latter study might have been due in part to the more prolonged period (two years) over which the experiment was conducted, but differences in rat strains could also be an important criterion. It may be seen from Table 13.2, that livers in which multiple foci of nodular hyperplasia were seen, were from animals which were killed at the termination of the experiment, and this would seem to indicate a gradual quantitative development of the lesions with time.

Cystic bile duct adenomata were also observed in the liver of two animals. Neither of the above reported studies by Terracini and Magee (1964) or Terracini (1969) found tumours of the intrahepatic bile ducts.

The telangiectatic-like lesions, seen as engorged dilated cavernous sinusoids were also, it would seem, induced by DMN treatment. It is not certain how these lesions arose but they presumably could have been formed through loss of hepatic cells brought about by the initial necrotoxic effect of DMN, or to loss of cells at a later stage of the experiment from some unknown cause.

Tumours of the kidney were found in 11 out of the 24 animals and were bilateral in five of these animals. Generally, unaffected kidneys and kidney remnants attached to tumours showed more pronounced chronic nephritis than in those animals which had two complete functioning kidneys. The finding of gross nephrocalcinosis in one kidney is also

worthy of comment in that from my own experience this type of lesion usually occurs spontaneously in the tubules of the cortico-medullary region. The pathogenesis of this change, found in the tubules of the outer cortex in this instance, is not understood.

The origin and nature of the tumours induced in the kidney by DMN treatment are more problematical than those of the liver. In the work of Terracini (1969) and Terracini and Magee (1964) which has already been mentioned above, the induction of a high incidence of renal tumours was seen in rats which had been previously injected with DMN within 24 hours of birth. The site of necrotoxicity and the major site of tumour induction by DMN in the adult rat was initially shown to be in the liver as demonstrated by the early work of Barnes and Magee (1954) and Magee and Barnes (1956). It has since been shown, however, that this spectrum of tumour induction can be altered by varying the dosing routine which in turn alters the extent of exposure and metabolism of the compound by tissues other than the liver (Magee and Barnes, 1967). There is now considerable evidence that it is the formation of an active metabolite which is necessary for carcinogenesis. The liver has been shown to be the principal site of breakdown of the compound followed in turn by the kidneys and then the lung (Magee and Vandekar, 1958; Magee and Barnes, 1962). The active carcinogenic metabolite has been suggested to be an alkylating agent of nucleic acids (Magee and Farber, 1962; Lee and Spencer 1964; Magee and Barnes, 1967; Lee and Goodall, 1968). It is relevant therefore that the highest levels of alkylation of DNA have been found in the liver as well as significant

but considerably smaller levels of alkylation in the kidney (Magee and Hultin, 1962; Craddock and Magee, 1963). Further evidence supporting the view that DMN requires metabolism to form the active carcinogen comes from experiments concerned with the administration of DMN to rats maintained on a protein deficient diet. When protein deficient diets are fed to rats, a single dose of 60 mg DMN/kg body weight will induce tumours of the kidney in virtually 100% of the survivors of the treatment (Swann and McLean, 1968; Hard and Butler, 1970a). Swann and Magee (1968) found that such a dietary restriction resulted in a 60 percent reduction in the metabolism of dimethylnitrosamine by liver slices while metabolism by kidney slices was not significantly altered. Furthermore, methylation of kidney DNA is increased under these circumstances. Thus it has been suggested that this decreased metabolism of DMN by the liver allows the compound to be present in the blood for a longer period than in normally fed rats. This in turn allows a more prolonged exposure of the kidney to DMN and an increased incidence of tumours in this organ thus ensues (McLean and Magee, 1970). In a similar way kidney tumours may be induced in a high percentage of rats surviving a relatively high dose of DMN. For example Jasmin and Riopelle (1968) reported that oral administration of 8 mg DMN/kg body weight/day for 6 consecutive days results in an over 80% incidence of kidney tumours. Magee and Barnes (1959; 1962) also reported that a single per os dose of 30 mg/kg induced tumours in 20% of surviving rats. In these cases the relatively high dosages presumably allowed more of the compound to reach the kidney.

The above findings on adult rats would also have an important bearing on the experimental carcinogenic findings of this chapter. Terracini and Magee (1964) found that the newborn rats, within the first 24 hours of life, were in fact capable of metabolising DMN. As in the adult rat, the unchanged molecule had completely disappeared from tissues by 24 hours, but nevertheless the metabolism was significantly slower over the first 12 hours in the young animal. Moreover, Lee and Spencer (1964) reported that over the first three days of life alkylation of kidney RNA was higher than in liver. Chapter 5 showed that the acute necrotoxic action of DMN (which has also been postulated to be due to an active metabolite; Heath, 1962) was patchy and irregular in nature in the 3-day old animal, in contrast to the uniform centrilobular necrosis brought about in adult rat liver. This was suggested, in the discussion, to be due to incomplete development of drug-metabolising enzymes by two days of age. Furthermore, although it would appear that the processing enzymes necessary for the metabolism of DMN are not part of that system responsible for carbon tetrachloride metabolism (McLean and Magee, 1970), the development of susceptibility of the neonate rat to the latter compound might be taken as further evidence of development of microsomal processing enzymes during the first few days of life. Biochemical and pharmacological studies also support this view of enzyme development (Jondorf et al, 1958; Fouts and Adamson, 1959; Kato et al, 1964) over the first few days from birth. In the light of the above evidence it is therefore not surprising that a single necrotoxic dose of DMN

administered at two days of age should give rise to the induction of a high incidence of kidney tumours. Fifty percent (12/24) rats in experiment 13 above developed renal tumours of which six showed tumours in both kidneys. All but one of these growths were found to be composed of mainly mesenchymal tissue and appear to conform to the early description of these tumours by Terracini and Magee (1964) who referred to them as anaplastic renal tumours. As such they consist of predominantly mesenchymal tissue within which is often isolated, remnants of frequently cystic and/or hyperplastic tubular epithelium and glomeruli. These mesenchymal cells in the kidney tumour appear to be capable of developing along one of several cell lines (in this particular study, smooth muscle, fibrous or myxoid connective tissue). The behaviour of these tumours can vary, some growing extremely rapidly and reaching very large proportions while others would seem to grow at a far slower rate.

The nature of the renal tumours induced by DMN administration to the adult rat has been extensively studied and it would seem to be appropriate at this point to discuss these tumour findings and current views on their histogenesis in the light of the neonate studies described above.

From a survey of published reports the kidney tumours which have been induced by administering DMN to the adult rat, would appear to fall in to one of two main categories. There are firstly those with a predominantly epithelial cellular constitution; the general consensus of opinion is that these growths arise from epithelial cells of the tubules and are thus adenomatous or carcinomatous in nature.

(Zak, 1960; Magee and Barnes, 1959; Riopelle and Jasmin, 1969; Hard and Butler, 1970a). The renal tumours which were labelled sarcomatoid in the early work of Magee and Barnes (1962) have attracted a more diverse nomenclature however e.g. nephroblastoma (Riopelle and Jasmin, 1963; Jasmin and Riopelle, 1964; Taper, 1967; Hadjiolov, 1968), anaplastic epithelial tumour (Zak, 1960), Wilms tumour (Argus and Hoch-Ligeti, 1961), embryonal cell tumour (Ito et al, 1969), renal sarcoma (Yang, 1966) stromal nephroma (Riopelle and Jasmin, 1969) and mesenchymal tumours (Hard and Butler, 1970a). Thus there is plainly a diversity of opinion with regard to the histogenesis of these growths. From the description given by the majority of the above authors, however, it may be concluded that these tumours were made up of mainly mesenchymal tissue containing one or a mixture of fibroblastic, myxoid, smooth muscle, primitive skeletal muscle, chondrocytic or osteogenic connective tissue. The most recent view is that these tumours are in fact mesenchymal in origin, are in most cases highly invasive and thus, in the process of their extension, isolate tubules and glomeruli, many of which may then become cystic and hyperplastic. (Jasmin and Riopelle, 1969; Hard and Butler, 1970a). Such a conclusion is in agreement with the observations on the renal tumours found in the experiment of this chapter.

Against this view are the proponents of a nephroblastic origin of these growths (Taper, 1967; Hadjiolov, 1968; Argus and Hoch-Ligeti, 1961; Ito et al, 1969). These workers advocate that the tumours are similar to the nephroblastoma or Wilms tumour seen in children. Hard

and Butler (1970a) were of the opinion however that the proponents of the nephroblastoma origin of these tumours were confusing the palisade-like and rosette patterns, occasionally adopted by fibrosarcomatous cells, with primitive nephrons, and wrongly identifying focal proliferation of dense nests of tumour cells indenting the lumen of dilated tubules as primitive glomeruli. Both these features were to be seen in the series of mesenchymal tumours induced in experiment 13 above and it is concluded that the explanation by Hard and Butler 1970a is the more likely.

The work of Hard and Butler (1970b), in which interstitial renal lesions in the region of the glomerular hilus were observed within a few days administration of DMN to protein deprived rats, has already been referred to in Chapter 12. These authors have postulated that it is these specific interstitial areas which give rise to eventual tumour formation. Furthermore in this and another study (Hard and Butler 1970a) these workers emphasize the vascular nature of the mesenchymal tumours induced by DMN and also the large amounts of smooth muscle found in them. On this evidence it was suggested that the DMN-induced mesenchymal tumours originate from the primitive mesenchymal cells (vascular pericytes) which normally would develop into mesangial cells of the glomerulus or the smooth muscle cells of the arterioles.

The tumours seen following the dosing of the neonate rat with DMN did not, as evidenced by the early neoplastic lesions described in Chapter 12, indicate which cell type or types were responsible for the tumour formation. On subjective analysis however it would seem that many of these tumours following neonatal administration of DMN could have arisen from primitive interstitial cells just below the cortico-

medullary junction. Of course it is important to emphasize that the two differing experimental procedures (administering DMN to protein deprived adult rats or to neonatal rats) would not necessarily lead to similar quantities of the carcinogen reaching the various zones of the kidney. The differential ability of susceptible cells to metabolise the carcinogen under these two different dosing routines and in these two ages of rat would also be relevant to the site of tumour induction.

If these mesenchymal tumours do in fact arise from a primitive mesenchymal cell at either site in the kidney, it would seem unnecessary to classify them further as did Riopelle and Jasmin (1969). It would seem more logical, in the interest of clarity and simplicity, to designate them as arising from primitive mesenchymal cells which are capable of developing along one of several cell lines. Mesenchymal tumours thus occur which may be solely, predominantly or a mixture of, fibrous, myxoid, chondrocytic, osteocytic connective tissue or smooth or primitive skeletal muscle. Accordingly these growths exhibit varying rates and patterns of growth.

The experiment in this chapter has thus shown carbon tetrachloride to be non-carcinogenic following single administration to the neonate rat. Under the same dosing regimen however DMN is a potent carcinogen producing almost exclusively, in this series, tumours in the liver and kidneys, none of which metastasized. The histogenesis of the much discussed mesenchymal tumours of the kidney is suggested, under this particular neonatal dosing regimen, to be from primitive mesenchymal cells in the cortico-medullary region.

ILLUSTRATIONS

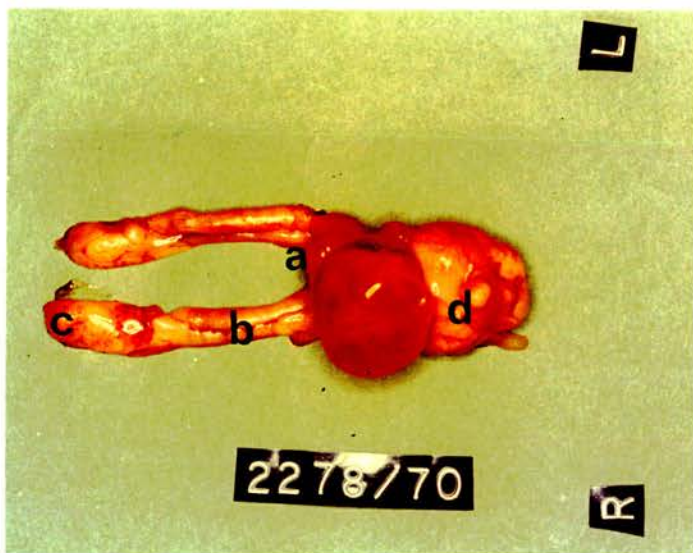
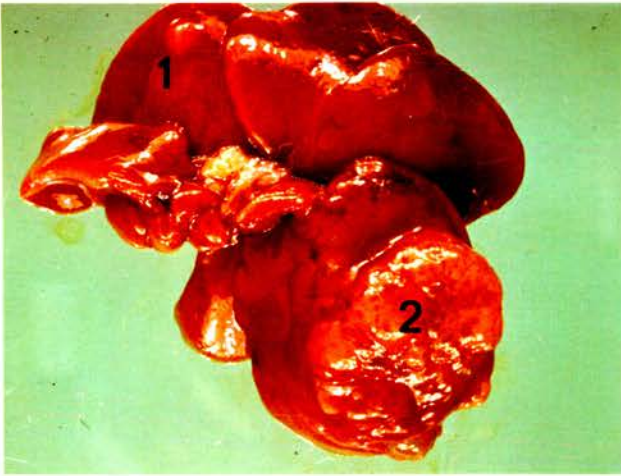


Fig. 13.1 Macroscopic appearance of vaginal fibroma found in a 65-week old control rat.
a. bladder; b. uterus; c. ovary; d. tumour.



Fig. 13.2 Macroscopic appearance of liver of rat treated with 10 mg DMN/kg body weight when two days of age and killed 17 months later. The dark red depressions over the surface of all the lobes were a common finding following this treatment.

(a)



(b)

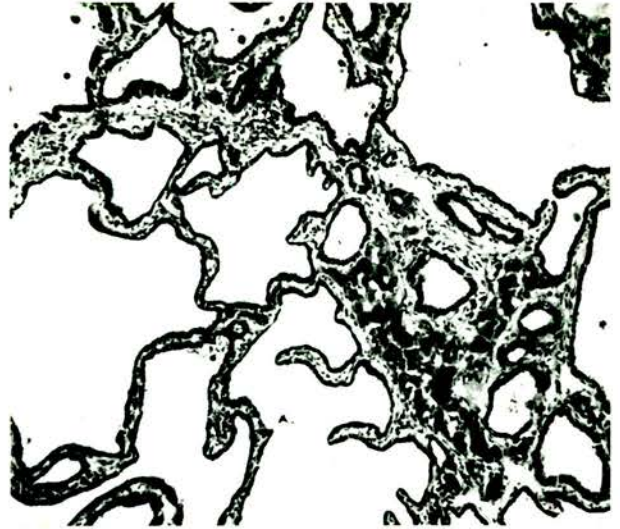


Fig. 13.3a Macroscopic appearance of liver of rat treated at two days of age with 10 mg DMN/kg body weight and killed after 14 months. This liver shows a small nodule, 1., which was composed of hepatocytes (see Fig. 13.4 below) and a large cystic bile duct adenoma, 2., seen microscopically in Fig. 13.3b above (H+E x 90).

(a)



(b)

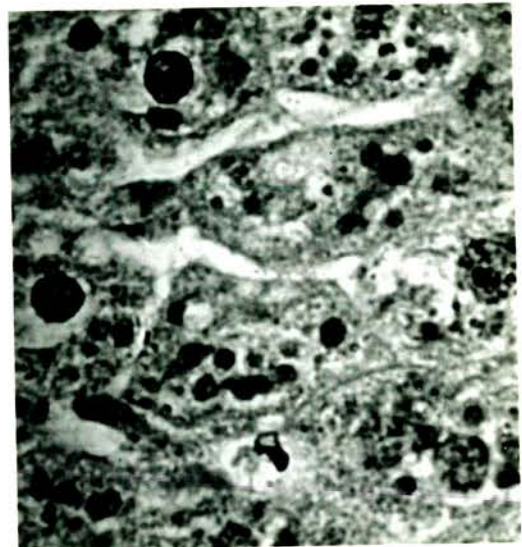


Fig. 13.4a Microscopic appearance of hepatocellular nodule of hyperplasia showing narrow zone of compressed cells at edge of the lesion (H+E x 90).

b) Large basophilic cytoplasmic inclusion bodies seen in the hepatocytes in areas of nodular hyperplasia in two animals (H+E x 880).



Fig. 13.5 Hepatocellular adenoma found in liver of rat injected at two days of age with 10 mg DMN/kg body weight and killed after 17 months.



Fig. 13.6 Histological appearance of area of above tumour showing hepatocytes in bizarre arrangement with grossly vacuolated cytoplasm (H+E x 360).

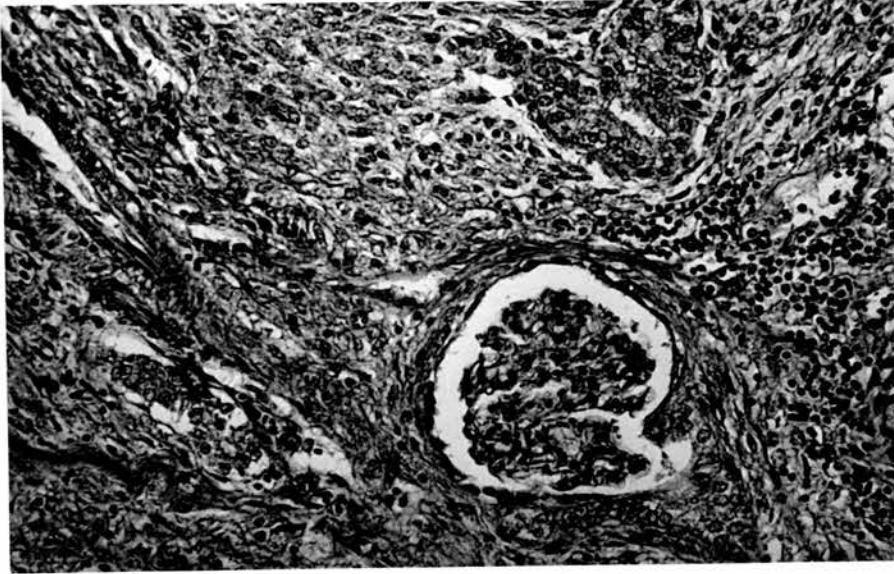


Fig. 13.7 Relatively intact glomerulus surrounded by mesenchymal tumour cells. Above glomerulus is a remnant of a tubule with hyperplastic epithelium. Animal treated 17 months previously with 10 mg DMN/kg (H+E x 220).

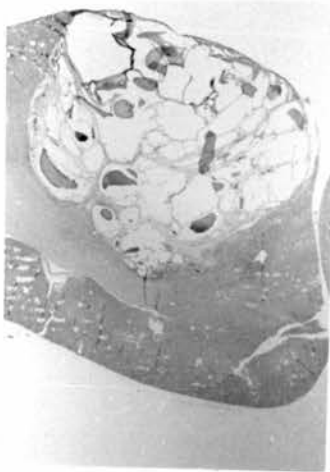


Fig. 13.8 Mesenchymal tumour and large cystic spaces in kidney of rat treated 14 months previously with 10 mg DMN/kg body weight.

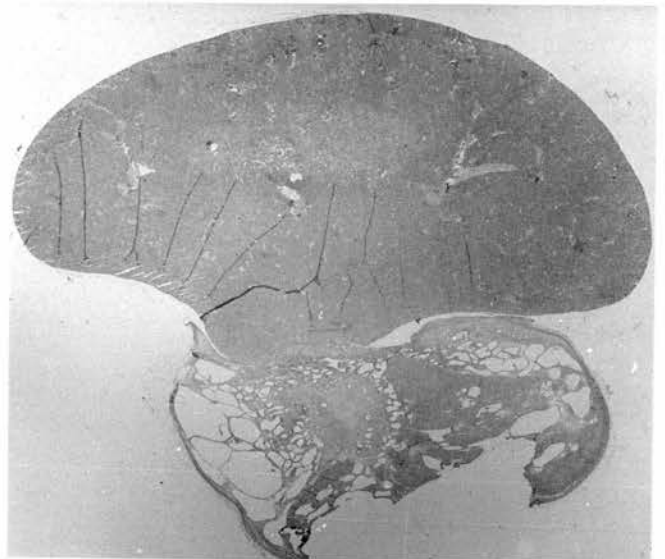


Fig. 13.9 Mesenchymal tumour projecting from the pelvic region of the kidney. This animal was treated with 10 mg DMN/kg body weight 17 months prior to being killed.

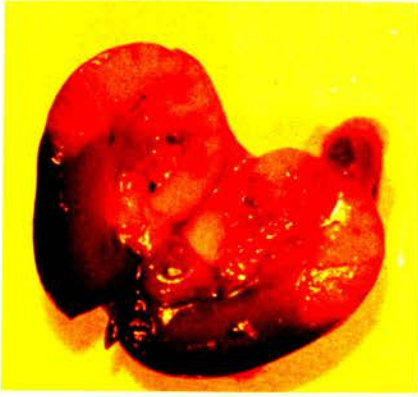


Fig. 13.10 Gross appearance of mesenchymal tumour of intermediate size which occupied approximately $\frac{2}{3}$ of the kidney but produced very little increase in the size of the organ. This rat was killed at 12 months of age having been injected at 2 days of age with 10 mg DMN/kg/body weight.



Fig. 13.11 Histological appearance of part of above tumour with remnants of tubules surrounded by smooth muscle. (Masson T. x 360).

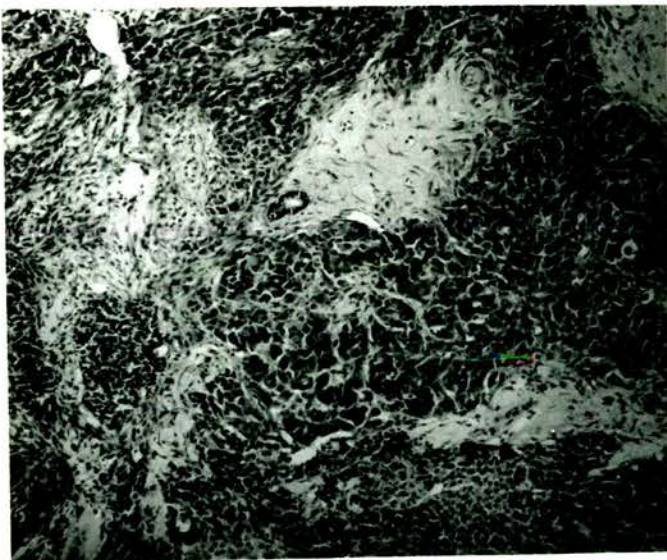


Fig. 13.12 Area of above tumour showing adenomatous growth (H+E x 90).

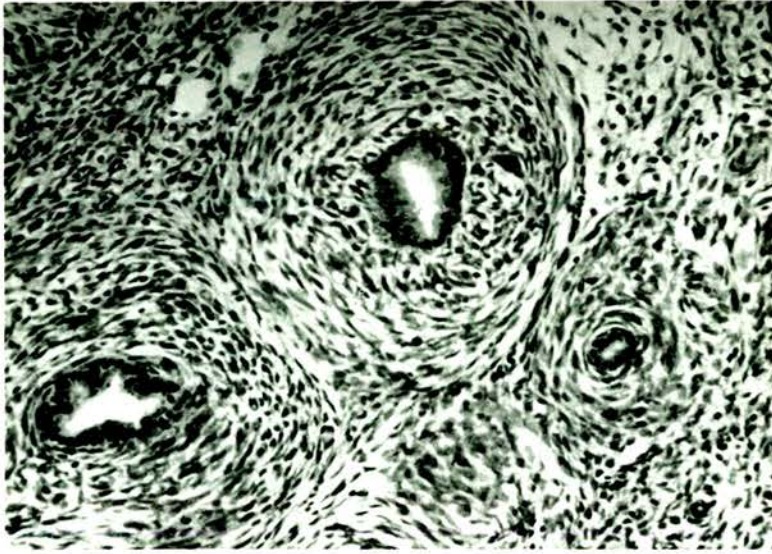


Fig. 13.13 Section of tumour where collecting tubules in the pelvic region of the medulla are surrounded by whorls of neoplastic mesenchymal cells (H+E x 220).



Fig. 13.14 Macroscopic appearance of the largest kidney tumours seen. The left kidney was hard and firm while the right was soft and very haemorrhagic. This rat was killed 13 months after administration of DMN.



Fig. 13.15 Example of the largest type of tumour encountered, all of which considerably distended the abdomen.

CHAPTER 14
CONCLUDING DISCUSSION

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At the conclusion of each of the relevant chapters of this thesis, there has been a discussion of the results of the experiments described. While many of the findings have thus already been dealt with in sufficient detail a final appraisal of the major aspects of the work is necessary. The nature of pathological nuclear enlargement via mitotic inhibition and the association of this phenomenon with carcinogenesis are two particular aspects of the work which provide an opportunity for further discussion.

1. Summary of major findings

The most important findings which have emerged from this work are as follows:

It was shown in Chapter 8 that a single dose of DMN to the 2-day old rat brings about a rise in nuclear ploidy in a large percentage of hepatocytes through an inhibition of mitosis following DNA replication (that is, the block occurs in G_2 of the cell cycle). Following administration, this antimitotic effect would appear to persist for approximately four days, after which time the affected hepatocytes are able to pass through succeeding mitoses in a normal fashion (see Chapter 8 and Chapter 11). The number of hepatocytes involved in such ploidy increases is dependent on the number of cells entering DNA synthesis (Chapter 11). Megalocytosis may be induced by continued administration of DMN so that many hepatocytes are blocked in the G_2 phase of successive cell cycles (Chapter 11).

Short and long lived populations of parenchymal cells exist in the liver and, within the latter group, the hyperdiploid cells induced by

the DMN treatment have an average generation time of longer duration than those hepatocytes of appropriate control livers (see Chapter 12).

In contrast to these findings, carbon tetrachloride and thiocetamide (Chapters 9 and 10) caused an increase in nuclear ploidy through an intermediate binucleate mitotic system.

There was no evidence of any other mechanism of pathological increase in nuclear ploidy in neonate liver, than the two described above.

In the carcinogenic study involving single administration of carbon tetrachloride or dimethylnitrosamine shortly after birth (Chapter 13) the latter compound was found to be highly active in this respect and induced tumours of mainly the liver and kidney. Carbon tetrachloride on the other hand was found to be entirely inactive as a carcinogen in this particular dosing regimen.

2. Mechanisms by which chemical and physical hepatotoxic treatments might interfere with the cell cycle

A. Interaction with DNA

The evidence concerning the interaction of DAB, aflatoxin and lasiocarpine with DNA has been briefly reviewed in Chapter 2. Alkylation of DNA, specifically the guanidine moiety, has also been demonstrated with DMN (Magee and Hultin, 1962) and this interaction has been postulated to be the underlying lesion initiating carcinogenesis by nitroso compounds (Magee and Farber, 1962; Lee and Spencer, 1964; Magee and Barnes, 1967; Lee and Goodall, 1968). It has also been suggested by other workers that specific damage to DNA by pyrrolizidine alkaloids could result in increased nuclear ploidy. It would therefore

seem worthwhile at this point to discuss in more detail the concept that damage to DNA might, in addition to delayed carcinogenic effects, interfere with the regulation of the cell cycle and induce immediate alterations in cellular kinetics.

Culvenor et al. (1962) first suggested the possibility that the ability of the toxic pyrrolizidine alkaloids to inhibit mitosis might lie in their alkylating potential. Jago (1969) enlarged on this concept of interaction of the alkaloids with DNA and still more recently McLean (1970) has suggested a model to explain how such damage could interfere with the cell cycle. Briefly reiterating the knowledge to date on which this postulate is founded, it has been shown that the pyrrolizidine alkaloids are metabolised in vivo to pyrroles (Mattocks, 1968; Culvenor, 1969). These authors showed that pyrroles are potentially capable of bifunctional alkylation of DNA. Specifically one of these pyrrolizidine alkaloids, lasiocarpine, has been shown to be capable of alkylating DNA in vitro (Culvenor et al., 1962) and more recently experiments have suggested that this also occurs in vivo (Culvenor et al. 1969). Moreover well known bifunctional alkylating agents such as mitomycin c and nitrogen mustard have been suggested to inhibit division by bringing about interstrand cross-linkages on the DNA molecule (Szybalski and Iyer, 1967; Lawley et al., 1969). In Table 3.1 of this thesis there are grouped together a number of chemicals in addition to the pyrrolizidine alkaloids which have the ability to block the initiation of mitosis of hepatocytes. A consideration of the nature of the damage to DNA and its effect on the cell cycle would therefore seem to be appropriate with each of these toxic chemicals and radiation damage.

i) Nature of damage to DNA brought about by hepatotoxic agents

There would appear to be differences in the nature of the damage to DNA brought about by the hepatotoxic agents under consideration. The interference with the cell cycle by radiation damage has been extensively studied and past work has been recently reviewed by Kanazir (1969). In vitro studies have shown that a significant decrease in viscosity of isolated DNA occurs after ionizing radiation due to a decrease in its molecular weight. This decrease has been observed both in vitro and in vivo in experimental systems utilizing bacteria and cells of different tissues of higher organisms. Ultracentrifugal analysis of irradiated DNA samples has demonstrated that irradiation produces both single and double strand breaks in viral, bacterial and mammalian DNA and that both partial and complete denaturation of the DNA molecule can occur. On this evidence Kanazir concludes that DNA is depolymerized by irradiation both in vitro and in vivo and cites further evidence that DNA accumulates far more single strand breakages than double strand breakages. These changes may also be accompanied by destruction or alteration of DNA bases causing branching and/or cross-linking of DNA. As will be discussed further, mammalian cells appear to have efficient processes for repair of damage to DNA (Setlow and Carrier, 1964; McGrath et al., 1965; Rassmussen and Painter, 1966; Lett et al., 1964; 1967).

The active metabolites of DMN responsible for the alkylation of DNA have been suggested to be either diazomethane or the highly reactive carbonium ions (Magee and Barnes, 1967). It seems unlikely

that DMN can effect cross-linkage of the DNA molecule (Magee - personal communication) and linkage of the two strands would not therefore appear to be implicated in its antimitotic action.

The binding of radioactive metabolites of DAB to DNA has been shown to be of a covalent nature (Roberts and Warwick, 1966; Warwick and Roberts 1967). The exact nature of the metabolite responsible for the interaction is not known however, and it is thus not possible to predict if cross-linking of DNA could occur with this compound.

Similarly, little is known of the nature of the damage to DNA brought about by aflatoxin, apart from the interaction between the two molecules. In summary, therefore, the pyrrolizidine alkaloids and to some extent radiation damage would both appear to be capable of cross-linking DNA; this would seem to be an unlikely effect resulting from the administration of DMN; knowledge on the interaction of aflatoxin or DAB with DNA is not sufficiently advanced to predict whether or not cross-linkage would occur.

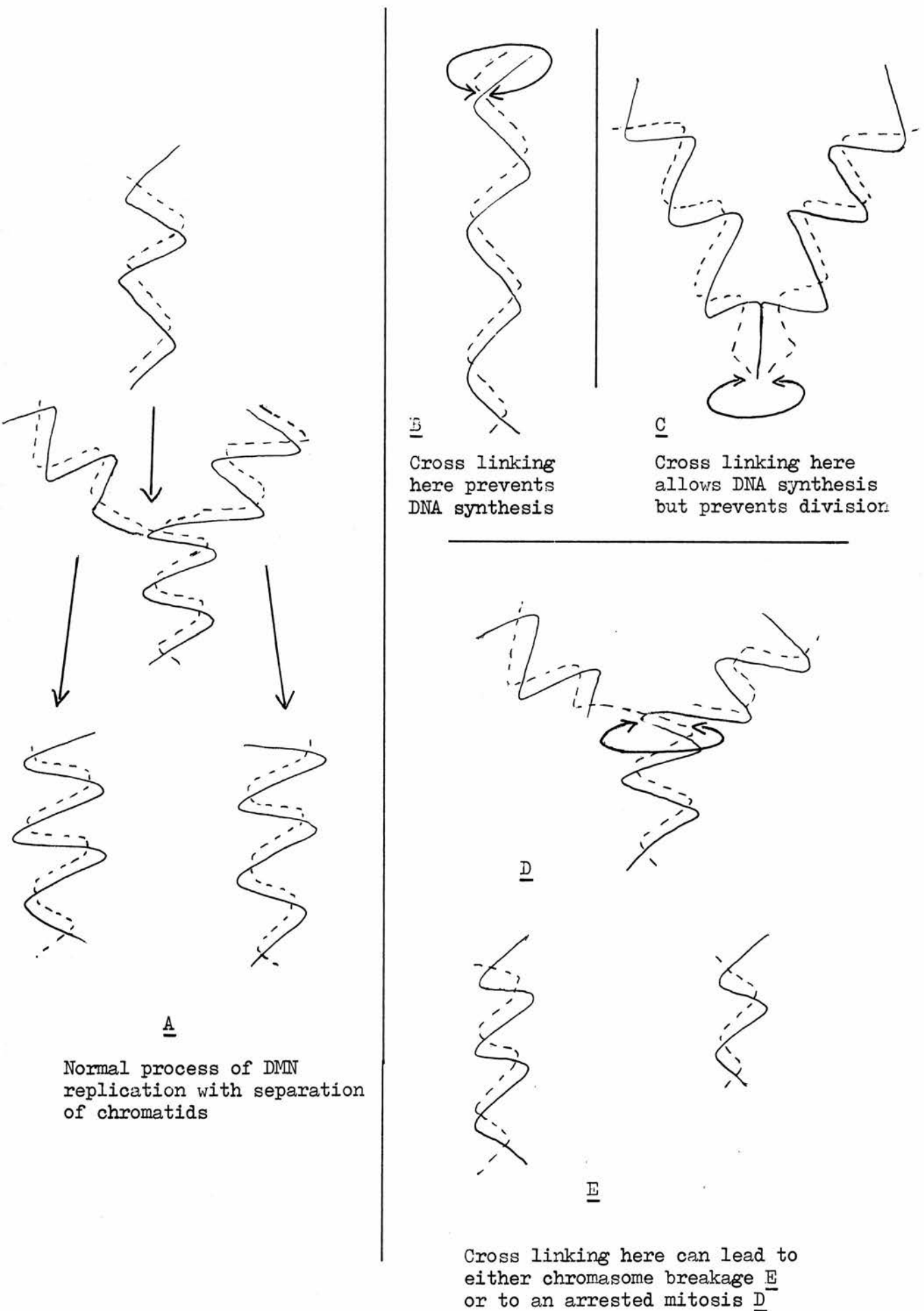
ii) Mechanisms by which damage to DNA could affect the cell cycle

As mentioned above, on the basis of the postulated alkylation of DNA, McLean (1970) proposed a model to explain some of the facets of liver poisoning by the pyrrolizidine alkaloids. This is reproduced in Text-Fig. 14.1. It is interesting to review how each of the above hepatotoxins fit into such a scheme of cell cycle changes.

a) Suppression of DNA synthesis

One possibility, is that the majority of the chromosomes are cross-linked across the major groove close to the site or sites at which unwinding starts (Fig. 14.1.B). DNA replication in this

Text-Fig. 14.1 The ways in which interstrand-cross-linkage of DNA might effect the process of cell division.



instance would be impossible.

There is considerable variability in the ability of the above hepatotoxins to suppress DNA synthesis. With lasiocarpine the subjective opinion has been formed that following a necrotoxic dose, the regenerative wave of DNA synthesis involving hepatocytes is unaffected (Jago, 1969). On the other hand a recent study by Frayssinet and Moulé (1969) has found that a single 30 mg/kg dose of lasiocarpine administered one or three hours before injection of tritiated thymidine leads to inhibition respectively of 70 and 90 percent of the incorporation of labelled thymidine in the twenty-four hour regenerating rat liver. These findings have been supported by Rogers and Newberne (1971) who also quantitated the effect of lasiocarpine on hepatocyte DNA synthesis during the reparative wave following partial hepatectomy. These authors reported that DNA synthesis was in fact considerably depressed twenty-four hours following the operation in rats injected three weeks previously with lasiocarpine (3 doses of 40 mg/kg on alternate days) when compared to appropriate control values. The most recent evidence therefore suggests that of the pyrrolizidine alkaloids, lasiocarpine in any event, is capable of depressing DNA synthesis in hepatocyte nuclei.

Radiation damage and its effect on the cell cycle has been extensively studied both in vivo and in vitro. In both of these experimental systems the effect induced depends very much on both the dose-rate and the stage of the cell cycle at which the cell is exposed. With the high degree of synchronisation obtained following partial hepatectomy, Fabrikant (1969) showed that both

depression and delay in the onset of DNA synthesis was most marked when the liver was irradiated at a time when the majority of the cells were in the late G₁ stage of the cell cycle. On the basis of observations of other workers, Fabrikant concluded that the time of irradiation following partial hepatectomy used in his own work (14 hours post operatively, 700 rads) would have had little effect on the level of enzymes such as DNA polymerase or thymidine kinase. In my own view this in itself could be interpreted as being indicative of a direct inhibitive action of irradiation on DNA replication. Such a concept is supported by in vitro studies. Irradiation of synchronised cultures of HeLa cells (Painter, 1962; Brent et al., 1966) has shown a similar depression of DNA synthesis although in this system it was the early stage of the S phase which was most susceptible to the effects. It was suggested by Brent et al. (1966) that the varying susceptibility of the cell to radiation damage at different stages of the cell cycle depended to some extent on the time available for repair of essential processes to take place before the onset of replication (and mitosis, see below). Such a repair system had been postulated earlier by Setlow and Carrier (1964) after detecting the disappearance of thymidine dimers from DNA of Escherichia coli after U.V. irradiation. Brent et al. (1966) also suggested that any repair system would require to eliminate the damage caused by irradiation in the presynthetic stages (in the time available before DNA synthesis) in order for normal replication to take place.

In a consideration of the effect of the remaining hepatotoxins on DNA synthesis, aflatoxin has been shown to depress DNA synthesis

for at least fifty hours following a necrotoxic dose in the rat (Rogers and Newberne, 1967). Experiment 8.4 in this thesis seemed to demonstrate a depression of DNA synthesis in the liver seven hours following administration of DMN and prior to a reparative wave of DNA synthesis. At the present time there would appear to be no knowledge of the effect of DAB or any of the closely related carcinogens on DNA synthesis.

Apart from DAB then, the above hepatotoxins have all been shown to have an inhibitory effect on DNA synthesis in the liver. In the instance of radiation damage at least, there is suggestive evidence that this might to some extent be due to the direct damaging effect on the integrity of the DNA molecule.

b) Mitotic inhibition

Leaving aside DNA synthesis, McLean 1970, proposed that where cross-linking is such that DNA synthesis is possible but separation of the chromatids is not, the cell will become a megalocyte (see Fig. 14.c). This thesis has already discussed in sufficient detail my suggestion that aflatoxin, DAB, the pyrrolizidine alkaloids and DMN bring about increased cell size through mitotic inhibition following DNA replication. The inhibitive effect of radiation on mitosis might usefully be elaborated on, since as with DNA replication, the degree of suppression could well depend on a repair system. Fabrikant (1969), in his studies on liver restoration described above, found that irradiation in G_2 of the cell cycle had by far the most pronounced effect on delay and suppression of mitosis when compared to irradiation during the G_1 and S phases. As G_2 was also the period during which

irradiation subsequently induced the greatest number of chromosomal defects it would seem reasonable to once again suggest that the time available for repair of DNA to be effected between exposure and mitosis, is a crucial factor.

c) Aberrant mitoses and mutation

The model proposed by McLean suggested that cross-linking of DNA could produce chromosomal defects by allowing separation of only some of the chromatids. Other chromatids which are bound too tightly to break would cause the cell to become fixed in mitosis so that it would ultimately die. Where the chromosomes break, and allow cell division to reach completion (Fig. 14.e) the mitotic figures will appear aberrant. Aberrant mitotic figures have been noted in the liver following administration of the pyrrolizidine alkaloid lasiocarpine, (Schoental and Magee, 1959), radiation damage (Albert, 1958) and DAB (Stitch, 1960). There is as yet no report of aflatoxin inducing chromosomal abnormalities in the liver in vivo, but such effects have been seen utilizing cultures of human leukocytes (Dolimpio et al., 1968). There would appear to be no reports of DMN enhancing the level of mitotic irregularities in the liver. A further effect of the chromosome breaks induced by these toxins would be that daughter cells would receive complements of DNA which differed from that of the parent cell. This type of lesion could then result in mutation. The pyrrolizidine alkaloids (Clerk, 1960; Avanzi, 1961; Alderson and Clark, 1966) radiation damage (Stromness and Kvellard, 1963; Brown, 1964; Newcombe and McGregor, 1965), aflatoxin (Legator, 1969) and DMN (Pasternak, 1962) have all been shown to possess mutagenic

potential. It should be pointed out however that such gross chromosomal defects are not necessary for a compound to express its mutagenicity. For example Lawley and Brookes (1961) suggested that the effect of alkylation of DNA at N₇ of the guanine moieties (as occurs with DMN) would be to weaken the bonding between cytosine and guanine. The hydrogen bonds between cytosine and the alkylated guanine would be reduced from two to one. This dissociation would allow thymine to base-pair with guanine and this in turn would profoundly alter the genetic coding of the molecule.

Thus there is at least circumstantial evidence that the effects of the above hepatotoxins on the cell cycle is due to damage to the DNA molecule. If this is so it would not seem to be valid to ascribe all the effects to cross-linkage however. The evidence at present is that the toxic pyrrolizine alkaloids (and to some extent radiation damage) could bring about such bonding of the two DNA strands. It is perhaps significant in this connection that only the alkaloids have a persistent antimitotic effect while in sharp contrast, DMN acts over one cell cycle only. Pursuing this concept as far as one is able, it is possible that cross-linkage will produce a persistent antimitotic effect but that other damage to DNA can lead to less permanent effects. This would mean that, to some critical degree, the integrity of both strands of DNA is necessary for mitosis to proceed normally. It would also infer that the damage to DNA is not repaired as efficiently in the sites of cross-linkage when compared to repair of monofunctional damage.

iii) Repair of DNA as an explanation of the
varying persistence of antimitosis

It will be remembered that in Chapter 8 it was suggested that following the administration of DMN most of the hepatocyte nuclei were affected by the toxic metabolite. Some of these cells which were in G_1 and are stimulated to enter DNA synthesis as part of the regenerative response to the DMN induced necrosis are unable to repair the damage (possibly including damage to DNA) in sufficient time to allow normal mitosis to proceed. As the time from administration of DMN lengthens, increasing numbers of cells are able to effect the necessary repair so that by day four of the experiment most are able to enter and complete mitosis. Similarly, those cells which are actually blocked in G_2 following DNA synthesis are able to repair the damage by the time of their next division and do so normally but at a higher ploidy level.

Such a repair process, as indicated above, does not preclude the possibility that DNA alkylation is the initial lesion causing this inhibitive effect on mitosis. Monofunctional alkylation of DNA (as would be induced by the suggested metabolites of DMN) is relatively non-toxic, as evaluated by the effect of such alkylating agents on micro-organisms (Brooks et al., 1969). This process favours a mechanism of single strand breakage (cf. radiation damage above) after which repair is very efficient (Strauss, 1969). The single strand breaks may be produced during alkylation or alkylated sites may be converted to single strand breaks by an endonuclease. It is possible, therefore, that the duration of the inhibition of mitosis by DMN is a function of the relative ability of the cell to repair the damage

to DNA prior to mitosis. Those cells which do not effect repair to the requisite degree are prevented in some way from entering mitosis. Stretching the potentiality of this mechanism as far as one is able, such a system would prevent damage to DNA and subsequent mutations being passed to the next cell generation before adequate repair had taken place.

On the other hand, the prolonged persistent antimitotic effect produced by pyrrolizidine alkaloids suggests that in the context of the induction of mitotic inhibition being through bifunctional alkylation, the repair of the damage to DNA is less efficient. In consequence the antimitotic effect is more permanent than in the case of monofunctional alkylation.

The observations of abnormal mitotic figures in megalocytes following cessation of administration of pyrrolizidine alkaloids might also be explained on the basis of failure to repair damage to DNA. With each successive period of DNA synthesis followed by mitotic inhibition, the size of the nucleus increases. This step-wise increase in DNA content will presumably result in dilution of the cross-linkage of DNA with the result that the cell might eventually be able to enter mitosis. Such linkages which remain however, would ensure that these mitotic figures appear abnormal on histological examination of the liver.

Although great emphasis has been placed on this thesis on interaction with DNA as the underlying cause of mitotic inhibition by the above hepatotoxins, it must be admitted that the evidence presented is

completely circumstantial. However, in the absence of specific knowledge on the processes involved in the initiation of mitosis it at least provides a working model. It is possible to suggest other cellular defects such as interference with protein synthesis which could lead to similar changes however.

B. Inhibition of protein synthesis

Throughout this thesis damage to DNA has been extensively discussed in relation to its possible effects on the cell cycle and, to a certain degree, other subcellular lesions have been ignored. One feature of hepatotoxicity which many agents share is the ability to inhibit protein synthesis. Recently, Verbin et al. (1969) suggested that inhibition of protein synthesis could be the cause of the prevention of hepatocytes entering both DNA synthesis and mitosis following the administration of cyclohexamide. It is unfortunate, however, that the experiments of Verbin et al. gave no indication of the degree of persistence of the block in G_2 of the cell cycle. It was seen in Chapter 8 of this experiment that DMN caused a permanent block in G_2 of a single cell cycle so that an irreversible shift in ploidy ensued. It will be seen from the discussion that now follows that cyclohexamide could, as is seen to some extent with radiation damage, cause merely a delay in the onset of DNA synthesis and mitosis. This delaying effect infers that the interference with the cell cycle is an arrest in G_2 rather than an absolute and permanent block. Indeed, a study on the effect of cyclohexamide on mitotic activity of intestinal cells has shown that in this tissue the antimitotic effect of the antibiotic falls off after six hours following a single dose and an approximately

synchronous wave of mitosis in epithelial cells then takes place (Verbin and Farber, 1967).

As outlined above, Verbin et al. (1969) also used cyclohexamide to investigate the effect of a known inhibitor of protein synthesis in the liver on the cell cycle of hepatocytes. Verbin and co-workers investigated the effect of this protein inhibition on the passage of cells through DNA synthesis and mitosis in the regenerating rat liver following partial hepatectomy. On the basis of previous reports that the peak in the wave of DNA synthesis occurs 20-24 hours post-operatively and the peak in the wave of mitosis occurs 24-30 hours post-operatively, these workers administered cyclohexamide by intraperitoneal injection at times when the majority of cells could have been expected to be in either G₁, S, or M of the cell cycle. To investigate the effect of cyclohexamide on DNA synthesis, Verbin et al. injected the antibiotic 22 hours after partial hepatectomy and killed the animal two hours later. The rats received an injection of thymidine-C¹⁴ twenty minutes prior to sacrifice. The uptake of thymidine, estimated on a pooled sample of nuclei was found to have been inhibited by approximately 95% when compared to appropriate controls. Mitosis of hepatocytes, as quantitated on histological sections, at 26 hours after the operation was inhibited completely whether cyclohexamide was injected at 14, 22 or 24 hours post-operatively. The experiments made no mention of increased ploidy or cell size and it should be stressed that all the above observations were recorded at single time interval only.

On this evidence it is clear that cyclohexamide has a marked effect on both DNA synthesis and the passage of cells through G_2 of the cell cycle in regenerating rat liver. The persistence of the arrest in the latter phase of the cell cycle remains unknown. Observations on the effect of the antibiotic on intestinal cells indicate that the interference with the cell cycle is a temporary one and the G_2 phase is merely prolonged. It would certainly seem, in the light of the effect of other hepatotoxins on protein synthesis, that this aspect of cyclohexamide hepatotoxicity must be very profound or specific if one is to explain the antimitotic effect of the antibiotic on this basis. Otherwise it would be difficult to explain the wave of mitosis following thiocetamide or carbon tetrachloride poisoning of the liver. Both of these hepatotoxins inhibit protein synthesis following administration (Barker, et al., 1963; Smuckler, et al., 1962) although it must be admitted that the persistence of this effect following a necrotoxic dose is not known in either case. However, one cannot exclude entirely the possibility that damage or depletion of protein can lead to irreversible shifts in ploidy. In this connection it is interesting that DMN not only alkylates DNA, but also RNA and protein. Thus, if damage to a specific protein or to synthesis of such a protein rather than a generalised depletion of proteins was capable of inducing mitotic inhibition, then interaction with DNA, RNA or protein could be the underlying antimitotic lesion in the case of this nitrosamine. Similarly, the azo-dye DAB (Rees and Varcoe, 1966; Warwick and Roberts, 1967) has been shown to interact with RNA and protein and

again, it is possible that one or a combination of these alterations is responsible for the antimitotic action of this compound.

3. Association of antimitotic activity with hepatocarcinogenesis

In Table 3.1 were grouped together three hepatotoxic substances (aflatoxin, DAB and the pyrrolizidine alkaloids) which on current knowledge appear to give rise to increases in nuclear ploidy via a process of mitotic inhibition. Aflatoxin, DAB and possibly the pyrrolizidine alkaloids are also capable of inducing hepatocellular tumours in the liver. On the basis of these two criteria of antimitotic and carcinogenic activity the work of this thesis has shown that dimethylnitrosamine should be included in this group of hepatotoxins. It is possible to elaborate a little further on the nature of the mitotic inhibition and its association with carcinogenic potential.

The most effective toxic substances inducing megalocytosis in the liver are without doubt the pyrrolizidine alkaloids with which single administration is sufficient to bring about a gradual increase in nuclear and cell size in a large proportion of the hepatocyte population (Schoental and Magee, 1957; 1959; Nolan, 1966; Jago, 1969). In spite of this persistent antimitotic effect however the tumour inducing potential of these alkaloids remains in doubt. (McLean, 1970). Some of this doubt derives from the fact that the antimitotic and necrotoxic effect of the alkaloids leads to gross distortion of liver architecture with subsequent difficulty in the differentiation between tumour foci and hyperplastic nodules. As McLean (1970) stressed, in the majority of the carcinogenic experiments with the pyrrolizidine

alkaloids which resulted in tumour formation, the dosing was interrupted or ceased altogether several months before death. In relation to this, if neoplastic change requires proliferation of hepatocytes one cannot expect such proliferation to appear while the potent antimitotic effect of continued dosing is present. A tumour study following single administration to the neonate rat might prove helpful in elucidating the more exact nature of their carcinogenic potential.

The remaining two substances in this group of mitotic inhibitors (aflatoxin, 4-dimethylaminoazobenzene) are indisputably potent liver carcinogens. However, the exact nature of their antimitotic effect requires more detailed appraisal. On current knowledge they would not appear to have the persistence of antimitotic activity exhibited by the pyrrolizidine alkaloids. An investigation similar to that carried out with DMN in the neonate rat might be of considerable value in elucidating the more precise nature of the action of these two compounds on the cell cycle. Similarly, such an investigation with AAF would also be very relevant to this problem of association of mitotic inhibition with carcinogenesis. Very little is known of the effect of this liver carcinogen on cell kinetics and a metabolite of the compound is known to interact with DNA.

In the present state of knowledge, however, it would seem reasonable to argue that toxic chemicals which bring about increased nuclear ploidy via mitotic inhibition in the liver are also likely to express a carcinogenic potential in the organ. Direct comparison of the appropriate pyrrolizidine alkaloids and DMN however, indicates that

a persistent antimitotic effect does not go hand in hand with increased carcinogenic potential. Indeed exactly the opposite would seem to apply with recovery of cells from the antimitotic effect being necessary for the carcinogenicity of the compound to express itself.

The one hepatotoxic treatment which does not fit neatly into the above concept is radiation damage. Although irradiation causes damage to DNA, is capable of suppressing mitosis and DNA synthesis and of inducing mitotic abnormalities and mutations, it is by no means a potent carcinogen in the liver. Maini and Stitch (1961) suggested that for a substance to be hepatocarcinogenic it must cause not only chromosomal damage but also cellular proliferation which, acting together, produce a genetically heterogeneous cell population from which tumour cells arise. Although this is somewhat of a generalisation it is possible that, while inducing mitotic inhibition in a manner similar to say DMN, radiation damage lacks the ability to induce an additional subcellular lesion capable of causing uncontrolled cellular proliferation in the liver.

Such a conclusion is born out by the experiments of Warwick (1967) in which administration of 2-methyl-4-dimethylaminoazobenzene required concurrent partial hepatectomy before the carcinogenic potential of the compound could be demonstrated.

The inference of this observation is that the underlying cause of carcinogenesis will not be found in a single sub-cellular lesion but in the interaction of several aberrant processes in the cell.

4. Mechanism of chemical carcinogenesis

It is possible that these aberrations leading to neoplasia could be derived from damage of multiple sites by the carcinogen. The alternative is that a single lesion to a particular constituent of the cell could lead to a sequence of changes in other sites and these changes in turn could lead to eventual neoplasia. The latter process is in many ways the easiest to investigate and interpret experimentally. Over the past ten years there has been amassed a large amount of experimental evidence of the interaction of carcinogens with DNA. Because neoplasia requires that uncontrolled proliferation be transmitted through succeeding generations of tumour cells it is hardly surprising that the genetic theory of cancer holds great credence among experimentalists at this time (Zimmerman, 1971). Zimmerman also pointed out that interference with repair mechanisms to genetic material could in itself mean that cells become very sensitive to genetic alternations. Such speculation suggests that genetic alterations and carcinogenesis are not only induced by agents acting directly with DNA. Apart from defects to these repair mechanisms it is possible that damage to RNA could lead to alterations in the sequence of DNA bases. 'Reverse transcriptase', which is able to synthesize DNA from and RNA template, has been found in oncogenic viruses (Chedd, 1971) and also in leukaemic cells and normal human foetal cells. Perhaps, then, it is more relevant to carcinogenesis that DMN, DAB, and AAF bind to liver RNA. This might also be true for the carcinogenic polycyclic aromatic hydrocarbons and the carcinogenic lactones which also interact with RNA (Brookes, 1971).

Finally, it would be misleading to leave this discussion on carcinogenesis with the impression that only cell constituents derived from the nucleus have been incriminated in this process. Zimmerman (1971) includes a suggestion in his publication that DNA of mitochondria could be involved in carcinogenesis and cites evidence of mutation of mitochondria by such carcinogens as N-nitro-methylurethane, 1-nitrosimidazolidone, 1-methyl-3-nitro-1-nitrosoguanidine and anthracene derivatives.

Leaving aside completely involvement of nucleic acids the suggestion of Harington (1967) may be cited as an example of an alternative postulate incriminating protein damage in carcinogenesis. The basis of this idea outlined by Harington, resides in the fact that there is considerable experimental evidence that relatively high intracellular concentrations of sulfydryl groups, in particular glutathione, are required in plants and animals for the normal sequence of mitosis and division. Thus, this worker goes on to suggest, carcinogenesis could begin with an interaction of the compound with the SH groups of an enzyme system directly associated with cell division so that an initial decrease leads to inhibition of division. The response of the cell to this inhibition could then be an adaptive adjustment of a feedback mechanism governing the synthesis of the inhibited SH system so that an excess of enzyme is ultimately produced and maintained. This in turn would lead to excess cell proliferation. The transformation from inhibition to over-synthesis of enzyme, and ultimately product, must be a stable one transmitted from mother to daughter cells. Although Harington acknowledges that this might involve

a disturbance in the metabolism of nucleic acids he also suggests that it could merely be an adaption at the cytoplasmic level brought about by activation of a gene rather than an alteration in structure.

Apart from the experimental work suggesting an intimate involvement of the level of sulphydral groups with cell division Harington cites a good deal of work on the effect of carcinogens on this cellular grouping. Neish et al. (1964), for instance, found correlations between the relative carcinogenic activity of azo-dyes and the ability of these compounds to increase the liver glutathione content. Similar areas of increased glutathione content have also been found in the skin following application of various carcinogenic anthracenes. Harington also lists a large number of carcinogens which have been shown to interact with sulphydral groups including lasiocarpine, N-alkyl-N-nitroso compounds and N-nitrosodialkylamines, and the carcinogenic azo-dyes.

It remains to be seen whether or not such rises in specific sulphydral groupings are the cause of cellular proliferation or simply accompany increased division. Suffice to say that a good deal of caution needs to be exercised in relating any single group of sub-cellular changes which appear to correlate with carcinogenic activity of compounds, with actual capability of inducing neoplasia.

5. Concluding remarks

By the use of a novel experimental system, the work of this thesis has clarified to some extent the nature of nuclear ploidy increases in the liver. Further investigation into the exact mechanism

of cell and nuclear enlargement following single administration of compounds such as aflatoxin, dimethylaminoazobenzene and acetylaminofluorene would yield valuable information. The neonate rat would seem to be an ideal experimental animal for this purpose with an almost homogenous diploid mononucleate hepatocyte population providing an excellent baseline from which development of cell populations is more easily followed. The increased susceptibility of the neonate animal to the carcinogenic action of hepatotoxic chemicals also provides an opportunity of correlating their action on the cell cycle with their potential for inducing neoplasia.

Hopefully, studies on cell kinetics coupled with advances on the biochemical and molecular effects brought about by toxic compounds will eventually lead to a fuller understanding of the control of cellular proliferation. Such an understanding is obligatory if a full appreciation of the sub-cellular mechanisms involved in carcinogenic processes is to be gained.

S E C T I O N V

APPENDIX

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Table 1

Differential mitotic counts/3000 hepatocyte nuclei in control and colcemid treated groups of 2-day old rats.

Dose level	Prophase	Metaphase	Anaphase	Telophase	Sex
0.02 mg/kg	1	4	2	1	M
	1	3	1	0	M
	0	5	1	0	M
	2	5	0	0	M
	0	4	0	0	M
	0	7	0	0	M
	1	7	1	1	F
	1	11	0	0	F
	0	6	0	0	F
	2	5	2	3	F
	0	5	0	0	F
	1	17	1	1	F
0.1 mg/kg	4	8	0	2	M
	0	11	0	2	M
	1	10	0	0	M
	0	13	0	1	M
	1	17	0	1	M
	2	15	0	0	M
	2	18	0	0	F
	1	13	0	2	F
	1	7	0	0	F
	1	16	2	1	F
	0	13	0	1	F
	2	19	0	0	F
0.2 mg/kg	3	16	1	2	M
	0	19	0	1	M
	0	12	0	0	M
	3	22	0	0	M
	0	11	0	0	M
	1	17	0	0	M
	0	24	0	0	F
	1	11	0	0	F
	0	8	0	0	F
	2	18	0	1	F
	1	12	0	1	F
	0	23	0	1	F

Continued/

Table 1 continued

1.0 mg/kg	2	10	0	0	M
	0	9	0	0	M
	0	6	0	1	M
	1	7	0	0	M
	3	26	0	0	M
	3	12	0	3	M
	2	8	0	0	F
	0	15	0	0	F
	0	7	0	0	F
	1	13	0	0	F
	2	28	0	0	F
	3	27	0	2	F
Control	2	1	0	0	M
	0	1	0	0	M
	0	4	1	1	M
	3	6	1	1	M
	7	2	0	0	M
	0	1	0	0	M
	2	1	0	1	M
	2	1	0	1	F
	2	2	0	0	F
	2	0	0	0	F
	0	1	1	0	F
	1	3	1	1	F
	2	0	1	0	F

Table 2

Counts of mononucleate and binucleate cells and isolated nuclei following varying times of disaggregation in TPB solution.

Time of disaggregation	Sample No.	Mononucleate cells	Binucleate cells	Isolated nuclei
10 minutes	1	15	8	60
	2	24	12	75
	3	25	11	54
30 minutes	1	485	219	831
	2	488	310	848
	3	492	260	740
60 minutes	1	962	491	977
	2	907	486	927
	3	923	466	733

Table 3

Number of nuclei in respective ploidy classes
in control and DMN treated animals injected at
two days of age and killed at varying times
thereafter.

Time interval in days follow- ing injection	Treated					Control				
	10 mg DMN/kg body wt.					20 µl saline/rat				
	Sex	2N	4N	8N	16N	Sex	2N	4N	8N	16N
1	M	78	22	-	-	M	95	5	-	-
	M	82	18	-	-	M	96	4	-	-
	F	87	13	-	-	M	98	2	-	-
	F	82	18	-	-	F	93	7	-	-
2	M	84	15	1	-	M	97	3	-	-
	M	63	36	1	-	M	93	7	-	-
	F	85	15	-	-	F	97	3	-	-
	F	68	31	1	-	F	96	4	-	-
3	M	65	32	3	-	M	97	3	-	-
	M	78	22	-	-	M	99	1	-	-
	F	61	39	-	-	F	98	2	-	-
	F	76	23	1	-	F	96	4	-	-
4	M	72	28	-	-	M	93	7	-	-
	M	59	40	1	-	M	97	3	-	-
	F	60	38	2	-	F	95	5	-	-
	F	60	38	2	-	F	95	5	-	-
5	M	64	36	-	-	M	98	2	-	-
	M	65	35	-	-	M	100	-	-	-
	M	65	35	-	-	F	99	1	-	-
6	M	84	16	-	-	M	96	4	-	-
	M	66	33	1	-	M	97	3	-	-
	M	64	36	-	-	F	97	3	-	-
	F	60	40	-	-	F	99	1	-	-
9	M	79	20	1	-	M	95	5	-	-
	M	68	31	1	-	M	99	1	-	-
	M	84	16	-	-	M	99	2	-	-
	F	77	23	-	-	F	100	-	-	-
14	M	70	22	7	1	M	97	3	-	-
	F	63	31	6	-	F	93	7	-	-
	F	76	22	2	-	F	93	7	-	-
21	M	85	15	-	-	M	97	3	-	-
	F	73	21	3	3	F	98	2	-	-
	F	66	27	5	2	F	98	2	-	-
	F	70	25	5	-	F	100	-	-	-
	F	74	21	4	1					

Table 4

Binucleate counts/2000 hepatocytes in control and DMN treated rats injected at 2 days of age and killed at varying times thereafter.

Time interval in days follow- ing injection	Control				Treated			
	20 µl saline/rat				10 mg DMN/kg body weight			
	M		F		M		F	
	Diploid	Hyper- diploid	Diploid	Hyper- diploid	Diploid	Hyper- diploid	Diploid	Hyper- diploid
1	8	-	8	-	15	-	8	-
2	3	-	9	-	6	-	7	-
	7	-	8	-	9	-	4	-
3	8	-	12	-	2	-	8	-
	6	-	10	-	7	-	14	-
4	3	-	4	-	6	-	4	-
	9	-	9	-	8	-	9	-
5	8	-	22	-	9	-	4	-
	6	-	11	-	16	-	16	-
6	7	-	3	-	13	-	12	-
	15	-	4	-	10	-	9	-
9	9	-	6	-	4	-	10	-
	14	-	24	-	14	1	16	-
14	18	-	22	-	9	-	6	-
	50	-	22	-	27	2	25	3
21	20	-	31	-	32	6	16	-
	212	-	192	-	124	26	182	19
	319	1	164	-	99	34	121	37

Table 5

Mitotic counts metaphase/3000 hepatocyte nuclei in control and DMN treated rats

Age in days	Control 20 μ l saline/rat		Treated 10 mg DMN/kg body wt.	
	M	F	M	F
1	10 10	22 32 45		
2	10 21 26	30 40 77 9		
2 1/4	43 21 46	33 38	8 6 1	4 1 0
3	41 9 41	15 21 11	10 3 6	9 7 0
4	17 11	16 24 15 17	6 1 10 3	15 3 2
5	11 19 9	27 40	6 14	10 7 15 27 7
6	8 25 9	14 19 6	12 15	7 34 45 21
7	11	16 38 19	3 37	7 22 16
8	22 16 13 24	11 15	14 18 23 20 23 19	28
9	10 55 24	21 12 20	9 7 30 35	11 51

Table 6

Counts of labelled nuclei/3000 hepatocyte nuclei in control and DMN treated rats administered tritiated thymidine.

Time interval following injection	Control		Treated	
	20 μ l saline/rat		10 mg DMN/kg body wt	
	M	F	M	F
7 hours	86	93	58	26
	66	59	42	40
1 day	76	81	153	123
	58	70	261	181
2 days	87	42	277	164
	66	80	139	208
3 days	82	133	100	84
		76	132	67
		56		
4 days	122	146	58	90
		87		
		81	97	57
5 days	63	81	95	62
	80	97	127 64	

Table 7

Mitotic counts/3000 hepatocyte nuclei in control
and CCl₄ treated rats injected at 4 days of age
and killed at varying times thereafter

Time interval following injection	Metaphase counts/3000 nuclei			
	Control		Treated	
	20 μ l saline/rat		0.1 ml CCl ₄ /kg body wt.	
	M	F	M	F
15 hours	8	11	14	14
	15	17	14	24
	12	13	10	20
1 day	18	10	142	157
	27	19	164	116
	17	19	141	111
2 days	9	22	48	37
	15	28	38	94
	31		34	90
	21			
3 days	22	13	16	10
	15	9	21	14
	11	19	11	41
4 days	53	17	26	20
	38	20	16	18
		13		17
		18		14

Table 8

Binucleate counts/2000 hepatocytes in control and CCl₄ rats treated at four days of age and killed at varying times thereafter

Time interval in days following injection	Control		Treated	
	20 µl saline/rat		10 mg CCl ₄ /kg body wt	
	M	F	M	F
1 day	5	8	9	8
	3	6	13	6
2 days	8	8	23	24
	4	10	27	24
3 days	6	3	16	33
	8	9	18	14
4 days	12	5	16	39
	13	9	21	20

Table 9

Metaphase counts/3000 nuclei in CCl₄ and control rats. Dosed at 28 days of age and killed at varying times thereafter

Age in days	Control		Treated	
	20 µl saline/rat		0.1 ml CCl ₄ /kg body wt	
	M	F	M	F
28	6	8		
	5	3		
29	3	2	27	17
	16	6	81	33
30	14	7	41	42
	4	6	45	60
31	8	15	14	12
	6	3	24	32
32	5	1	5	4
	7	1	2	7

Table 10

Binucleate cell counts/1000 hepatocytes following administration of CCl₄ to rats 28 days of age

Age in days	Control				Treated			
	20 µl saline/rat				0.1 ml CCl ₄ /kg body wt			
	Diploid	Hyper-diploid	Diploid	Hyper-diploid	Diploid	Hyper-diploid	Diploid	Hyper-diploid
	M		F		M		F	
28	242	-	261	4				
	285	3	297	1				
29	317	1	326	-	150	2	187	-
	252	-	275	3	176	-	192	1
30	247	1	277	3	138	-	34	-
	292	1	362	2	104	-	51	-
31	298	1	251	5	95	-	74	-
	235	-	276	3	113	-	33	1
32	293	1	265	-	50	-	174	2
	294	2	247	1	110	-	152	-

Table 11

The percentage differences between respective mean metaphase counts at each time interval in control and DMN treated rats

Age in days	Means of square roots of counts		
	Control	Treated	Difference
2 1/4	5.96	1.55	4.41 *
3	4.60	2.17	2.43 *
4	4.05	2.20	1.85 *
5	4.44	3.39	1.05
6	3.57	4.52	-0.95
9	4.48	3.82	0.66
14	4.07	4.53	-0.46
21	4.67	4.51	0.10

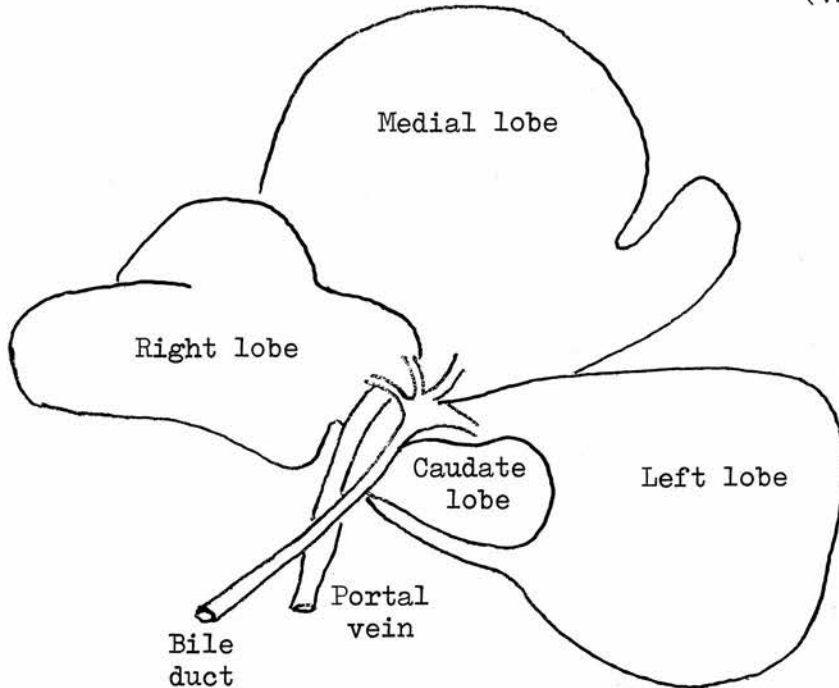
The average difference needed for significance is 1.4 (p<.05)*

B. Histological techniques

1. Sampling of portions of liver for histology.

Diagram of the anatomical structure of the rat liver.

(visceral surface)



In all experiments with rats up to 9 days of age, requiring histological appraisal only, the whole liver (apart from one 3 mm block retained for fat staining etc.) was embedded visceral surface down. The block was trimmed down until as large an area of all lobes of the liver apart from the caudate lobe was obtained on the section. A similar technique was utilised for preparing sections from neonate livers for evaluation of mitotic activity and DNA synthesis by autoradiography. Quantitative mitotic or autoradiographic appraisal of livers from rats older than 9 days was carried out on a single transverse section of the medial lobe only.

In Experiment 6.3 radial sagittal sections were taken of each lobe of the liver and particular care was taken to see that the section passed through the hilus of the organ so that an appreciation of the distribution of this lesion in relation to this area of the liver was obtained.

Where samples of liver were taken from animals up to three weeks of age for quantitative measurements requiring disaggregation (microspectrophotometry or binucleate cell counts) they were removed from the extremity of each of the three major lobes. The remainder of the liver was then embedded visceral surface down as before, for histology. In animals over 3 weeks of age 3 cm³ blocks of liver from the medial lobe were taken for disaggregation purposes.

2. Staining techniques

The following histological staining techniques were used in this work:-

<u>Stain</u>	<u>Demonstration</u>	<u>Reference</u>
Acid Fuchsin-Picric Acid	Collagen	Van Gieson I (1889)
Alcian-Blue Periodic Acid-Schiff	Mucins	Mowry (1956)
Carbol-Chromotrope	Eosinophils	Lendrum (1944)
Chloranilic Acid	Calcium	Disbrey and Rack(1970)
Haematoxylin and Eosin	Routine	Ehrlich (1886)
Methylene Blue-Aluminium Sulphate	Mast cells	Heath (1962)
Oil Red O	Lipid	Casselman (1959)
Phospho-Tungstic-Acid	Muscle striation	Lieb (1948)
Prussian Blue	Haemosiderin	Perls (1867)
Trichrome	Collagen and Smooth muscle	Masson (1929)
Silver impregnation	Reticulin	James (1967)

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